Characterization of the endogenous promoters in Yarrowia lipolytica for the biomanufacturing applications

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Abstract

Background: *Yarrowia lipolytica*, an oleaginous yeast with the GRAS status, has been developed as a platform for the chemical production. Specifically, promoter engineering is an important approach to regulating gene expression at the transcriptional level, which is of great significance in constructing microbial cell factories. Until now, some work on the promoter study has been carried out in *Y. lipolytica*. However, compared with other microorganisms, such as *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *etc.*, obtained achievements still need further replenishment and development for *Y. lipolytica*.

Results: To accurately analyze the promoter strength and avoid background interference, we used the NanoLuc luciferase reporter method. Furthermore, we screened 81 endogenous promoters in *Y. lipolytica*, mainly involved in carbon metabolism, amino acid metabolism, and lipid metabolism. Among them, the strongest promoter is P_{MnDH2} (*YALI0D18964g*), 1.60-fold of the strength of the P_{TEF} promoter, and the weakest promoter is P_{PHO89} (*YALI0E23859g*), 0.06% of the P_{TEF} promoter.

Conclusions: As a result, we obtained 15 strong promoters, 41 medium strength promoters, and 25 weak promoters, with the strength spanning from 0.06% to 1.60-fold of P_{TEF} promoter. In general, our study provides a unique and available endogenous promoter library for *Y. lipolytica*.

Background

Yarrowia lipolytica is an oleaginous yeast with the generally recognized as safe (GRAS) status, which is well known for its superior capacity for fatty acid synthesis[1, 2]. Specifically, its distinct endogenous metabolism, broad substrate spectrum, and robustness in the fermentation production have made *Y. lipolytica* as a potential organism for industrial applications[3–5]. Moreover, its relatively straightforward inherited background, well-developed genetic tools, and public availability of knockout collections, such as Po1 series, make it attractive as a metabolic engineering host[6]. Currently, *Y. lipolytica* has been successfully engineered to produce various natural and non-natural chemicals, including 2-phenylethanol[4], polyketides triacetic acid lactone[7], flavonoids[8, 9], erythritol[10], and so forth. In addition, *Y. lipolytica* is characterized by a solid ability to secrete proteins, thus it also has been developed as a platform for extracellular protein production[11].

Currently, the productive performances of strains could be optimized by rewiring the intracellular metabolic network[12]. It is worth noting that promoter engineering is an essential means to regulate and influence the timing and pattern of gene expression at the transcriptional level, further affecting the metabolic activities of microorganisms[13, 14]. Therefore, the promoter study is of great significance in metabolic engineering and synthetic biology. In *Y. lipolytica*, the promoter of translation elongation factor EF-1α, namely P_{TEF}, is a strong constitutive promoter, which is widely used in the research of gene expression[9] and cell factory construction[15]. Furthermore, the promoter P_{TEF} has also been used to construct the artificial hybrid promoters to enhance the transcription strength or endow the inducible properties[14]. For example, Blazeck et al. truncated the P_{TEF} sequences into multiple regions to connect

with the upstream activating sequences (UAS), and found that controlling the number of UAS in series within 8–16 can significantly improve the activities of hybrid promoters[16]. Besides, researchers have characterized several endogenous promoters in *Y. lipolytica*. For example, Juretzek et al. analyzed the intensity and induction effect of endogenous promoters P_{G3P}, P_{ICL1}, P_{POT1}, P_{POX1}, P_{POX2}, and P_{POX5} under different carbon source conditions[17]. Liu et al. characterized 22 promoters in the lipid metabolism to gain a deep understanding of lipogenesis in *Y. lipolytica*[18]. Although some work on the promoters has been carried out, compared with other microorganisms, such as *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *etc.*, achievements still need to be further replenished and developed for *Y. lipolytica*. Moreover, the relatively few available promoters are challenging to meet the requirement of metabolic engineering for constructing microbial *Y. lipolytica* factories.

Herein, we screened 81 endogenous promoters in *Y. lipolytica*, mainly involved in carbon metabolism, amino acid metabolism, and lipid metabolism. To accurately analyze the promoter strength and avoid background interference, we used the NanoLuc luciferase reporter method[19]. As a result, 15 strong promoters, 41 medium strength promoters, and 25 weak promoters have been characterized in this study. Among them, the strongest promoter is P_{MnDH2} (*YALI0D18964g*), 1.60-fold of the strength of the P_{TEF} promoter, and the weakest promoter is P_{PHO89} (*YALI0E23859g*), 0.06% of the P_{TEF} promoter, indicating that we obtained an endogenous promoter library with the strength spanning from 0.06% to 1.60-fold of P_{TEF} promoter. In general, our study provides a unique and available promoter library for studying *Y. lipolytica* cell factories, which will have great potential for industrial applications.

Results

Screening and characterization of the endogenous promoters in Y. lipolytica

Y. lipolytica has some unique metabolic advantages, such as the high metabolic flux of acetyl-CoA and citric acid. Therefore, focused on these metabolic pathways, including glycolysis, tricarboxylic acid cycle (TCA), pentose phosphate pathway (PPP), and fatty acid synthesis pathway, we selected 81 promoters for systematic analysis. To obtain complete promoter sequences, we truncated 1500 bp upstream before the ATG site of the corresponding gene through literature mining and the KEGG database.

Noticeably, a stable and reliable reporter system is essential for accurate analysis of promoter strength[20]. Currently, commonly used reporter systems for promoter analysis mainly include[19]: fluorescent proteins (generate fluorescence) and enzymes (generate chromogenic products), such as X-gluc (5-Bromo-4-chloro-3-indolyl-β-glucuronide). However, *Y. lipolytica* could generate severe fluorescence background[19], which interferes with the reliabilities of the fluorescent protein. On the other hand, the reporter system based on X-gluc also has several defects, such as being time-consuming, low efficiency, and labor-intensive. Specifically, Wong et al. developed a luciferase reporter system in *Y. lipolytic* with the advantage of being stable, efficient, and instant[19]. Therefore, we chose the luciferase reporter system developed by Wong et al.[19] for this study (Fig. 1).

We next verified the availability of the luciferase reporter system and characterized the strength of promoter P_{TEF} without intron (P_{TEF}). Consistent with the previous report[19], our results showed that promoter P_{TEF} is a typical strong constitutive promoter and its strength reached 4.31×10^7. For the convenient analysis of the experimental results, we used the promoter P_{TEF} as control and defined the strength of promoter P_{TEF} as 100, while classifying the endogenous promoters into strong promoters (that strength is higher than 50), medium-strength promoters (that strength is from 10 to 50), and weak promoters (that strength is lower than 10).

Carbon metabolism

Glycolysis pathway

In the glycolysis pathway, we analyzed 10 promoters, including P_{HXK1} (*YALI0B22308g*), P_{GPI} (*YALI0F07711g*), P_{PFK1} (*YALI0D16357g*), P_{FBA1} (*YALI0E26004g*), P_{TDH1} (*YALI0C06369g*), P_{PGK1} (*YALI0D12400g*), P_{GPM1} (*YALI0B02728g*), P_{ENO1} (*YALI0F16819g*), P_{PYK1} (*YALI0F09185g*), and P_{TPI1} (*YALI0F05214g*). As shown in Fig. 2, our experimental results showed that P_{HXK1}, P_{GPI}, P_{PFK1}, P_{FBA1}, P_{TDH1}, and P_{GPM1} are strong promoters, P_{ENO1}, P_{PYK1}, and P_{TPI1} are medium-strength promoters, while P_{PGK1} is a weak promoter. Among of these promoters, P_{TDH1} has the highest strength and reached $5.47\text{x}10^7$, which is 1.27-fold of the P_{TEF} promoter. The promoter P_{TDH1} is responsible for the transcription of glyceraldehyde 3-phosphate dehydrogenase TDH1, which catalyzes the conversion of glyceraldehyde 3-phosphate to 3-phospho-glyceroyl phosphate, indicating its important role to maintain glycolysis. Interestingly, Hapeta et al. identified that the hexokinase HXK1 is a rate-limiting step for converting glucose to glucose 6-phosphate in *Y. lipolytica*[21]. And, overexpression of HXK1 by the P_{TEF} promoter could significantly increase the carbon flux of glycolysis and improved lipid production[21]. It is reasonable because the transcriptional activity of P_{HXK1} is $2.16\text{x}10^7$, 50.2% of the P_{TEF} promoter. Moreover, the strength of other strong promoters P_{GPI}, P_{PFK1}, P_{FBA1}, and P_{GPM1} reached $3.26\text{x}10^7$, $3.00\text{x}10^7$, $2.37\text{x}10^7$, and $2.46\text{x}10^7$, respectively, which were 75.83%, 69.62%, 55.08% and 57.31% of the P_{TEF} promoter.

Most notably, the weakest promoter in glycolysis is P_{PGK1}, reaching $5.74\ \text{x}10^5$, which is 1.3% of the activity of the P_{TEF} promoter. The promoter P_{PGK1} is responsible for the transcription of phosphoglycerate kinase PGK1, which catalyzes 3-phospho-glyceroyl phosphate to 3-phospho-glycerate. However, the pathway analysis found that 3-phospho-glycerate could also be generated in the glyoxylate metabolism, suggesting that glycolysis is not the primary way for producing 3-phospho-glycerate in *Y. lipolytica*. In addition, the strength of medium-strength promoters P_{ENO1} (transcribing enolase ENO1), P_{PYK1} (transcribing pyruvate kinase PYK1), and P_{TPI1} (transcribing triosephosphate isomerase TIP1) reached $1.22\text{x}10^7$, $1.93\text{x}10^7$ and $1.65\text{x}10^7$, respectively, which were 28.24%, 44.71% and 38.27% of the P_{TEF} promoter.

Pentose phosphate pathway

The PPP pathway is the branch metabolism of glycolysis, which can completely oxidize glucose into 12 NADPH per glucose[5, 22]. Specifically, studies have demonstrated that NADPH supply is a rate-limiting step for fatty acid synthesis in *Y. lipolytica*, which affects the electron transfer efficiency to alter the titer and yield of fatty acid[5, 23]. However, *Y. lipolytica* owns multiple functional $NADP^+$-specific dehydrogenases, such as malic enzyme, isocitrate dehydrogenase, and glutamate dehydrogenase, which can complement the PPP pathway[18]. Nonetheless, Wasylenko et al. used ^{13}C Metabolic Flux Analysis to analyze strains with high/low fatty acid titer, and identified that NADPH for fatty acid synthesis is mainly supplied by the PPP pathway[24]. Herein, we investigated 6 promoters to understand the PPP pathway, including P_{ZWF1} (*YALI0E22649g*), P_{GND2} (*YALI0B15598g*), P_{SOL2} (*YALI0C19085g*), P_{RPE1} (*YALI0C11880g*), P_{RKI1} (*YALI0B06941g*), and P_{TKL2} (*YALI0D02277g*).

Concretely, the metabolic reactions with generating NADPH are catalyzed by glucose-6-phosphate dehydrogenase ZWF1 (transcribed by P_{ZWF1}) and 6-phosphogluconate dehydrogenase GND1 (transcribed by P_{GND2})[18]. Our results (Fig. 2) showed that the strength of P_{ZWF1} and P_{GND2} were $1.30x10^7$ (29.98% of the P_{TEF} promoter) and $8.27x10^6$ (19.21% of the P_{TEF} promoter), respectively, indicating that the intracellular NADPH can be increased by replacing the promoter. Consistent with our results, Yuzbasheva et al. enhanced the expression of ZWF1 to increase the carbon flux to the PPP pathway and improved the fatty acid synthesis[23]. Moreover, promoters P_{SOL2}, P_{RPE1}, and P_{TKL2} were medium-strength promoters, and their strengths reached $7.88x10^6$, $8.04x10^6$, and $8.74x10^6$, respectively, which were 18.31%, 18.69%, and 20.3% of the P_{TEF} promoter. Nevertheless, P_{RKI1} is a weak promoter with an activity of $2.86\ x10^6$, indicating that ribose 5-phosphate isomerase RKI1 is a rate-limiting step in the PPP pathway.

Pyruvate metabolism and tricarboxylic acid cycle

Pyruvate is the end metabolite of glycolysis, which could be oxidized to acetyl-CoA or converted to byproducts, such as acetate, ethanol, and lactate[25]. Here, we characterized 8 promoters in the pyruvate metabolism, including P_{LPD1} (*YALI0D20768g*), P_{LAT1} (*YALI0D23683g*), P_{PDB1} (*YALI0E27005g*), P_{PDC1} (*YALI0D06930g*), P_{PDC2} (*YALI0D10131g*), P_{ADH1} (*YALI0A15147g*), P_{ADH2} (*YALI0A16379g*), and P_{ADH3} (*YALI0F09603g*). As shown in Fig. 2, the promoter P_{LPD1} is the strongest promoter, reaching $2.80x10^7$, which is 56.58% of the P_{TEF} promoter. The promoter P_{PDB1}, P_{PDC1}, P_{PDC2}, P_{ADH2}, and P_{ADH3} are medium-strength promoters, and their strengths are $9.88x10^6$, $5.04x10^6$, $5.57x10^6$, $4.65x10^6$ and $1.07x10^7$, respectively, which were 22.96%, 11.73%, 12.95%, 10.80% and 24.96% of the P_{TEF} promoter. Moreover, the promoter P_{ADH1} is a weak promoter with a strength of $4.63x10^5$, indicating alcohol dehydrogenase ADH1 is not a primary alcohol dehydrogenase or a condition induced alcohol dehydrogenase in *Y. lipolytica*. Surprisingly, the transcriptional activity of the pyruvate dehydrogenase LAT1 promoter, P_{LAT1},

displays a low strength, reaching 2.53x10^6, 5.90% of the P_{TEF} promoter. This result is beyond our expectations because LAT1 is an indispensable step in the oxidative reaction of pyruvate to acetyl-CoA.

Undoubtedly, the TCA cycle plays a significant role in cellular metabolisms, such as maintaining energy metabolism and generating precursors for cell biomass synthesis[26]. We investigated the promoters of ATP citrate lyase ACL, citrate synthase CIT1, aconitate hydratase ACO1, and isocitrate dehydrogenase IDP2. The promoter P_{ACL} (*YALI0D24431g*) and P_{CIT1} (*YALI0E00638g*) are both medium-strength promoters and are responsible for the transcription of citrate lyase ACL and citrate synthase CIT1, respectively. Most strikingly, citrate lyase ACL and citrate synthase CIT1 both catalyze oxaloacetate to citrate. Specifically, it was reported that cellular AMP levels would decrease when nitrogen is depleted, further causing the decline of the isocitrate dehydrogenase activity to accumulate citrate for fatty acid synthesis[27]. Analogously, we found that the activity of P_{ACO1} (*YALI0D09361g*, driving the expression of aconitate hydratase to produce isocitrate) was significantly lower than P_{ACL} and P_{CIT1}, indicating the promoter P_{ACO1} is also strongly regulated by nitrogen starvation. In addition, the promoter P_{IDP2} (*YALI0F04095g*), driving isocitrate dehydrogenase, is a medium-strength promoter and displays an increasing transcriptional activity at the exponential stage.

Fatty acids synthesis

The accumulated citrate would be transported from mitochondria into cytoplasm for cleaving into acetyl-CoA[28]. Acetyl-CoA, a direct precursor, provides the basic building block for acetyl-ACPs synthesis[1]. Most notably, acetyl-ACPs are required to be transported into the endoplasmic reticulum for elongation or desaturation to synthesize fatty acids[1, 28]. Next, we investigated 20 promoters (Table 1) in the fatty acids synthesis metabolism. As a result, 1 strong promoter, 11 medium-strength promoters and 6 weak promoters were characterized.

Table 1
The characterized promoters in the lipogenic metabolism

Pathway	Name	Gene	Enzyme	Strength	Value
The fatty acids synthesis	P_{FAA1}	*YALI0B20196g*	fatty acid elongase	Weak	$4.05x10^6$
	P_{FOX2}	*YALI0C19965g*	3-oxoacyl reductase	Medium	$9.79x10^6$
	P_{ETR1}	*YALI0C19624g*	trans-2-enoyl-CoA reductase	Medium	$1.08x10^7$
	P_{ACL2}	*YALI0D24431g*	ATP citrate lyase	Medium	$2.01x10^7$
	P_{ACC1}	*YALI0C11407g*	acetyl-CoA carboxylase	Weak	$5.74x10^5$
	P_{FAS1}	*YALI0B15059g*	fatty acid synthase	Medium	$4.93x10^6$
	P_{FAS2}	*YALI0B19382g*	fatty acid synthase	Medium	$1.79x10^7$
	P_{SCT1}	*YALI0C00209g*	glycerol-3-phosphate acyltransferase	Medium	$7.86x10^6$
	P_{DGA1}	*YALI0E32769g*	diacylglycerol acyltransferase	Medium	$5.08x10^6$
	P_{MCT1}	*YALI0E18590g*	S-malonyltransferase	Weak	$2.71x10^6$
	P_{PPT}	*YALI0F14135g*	palmitoyl-protein thioesterase	Weak	$5.64x10^5$
	P_{ELO1}	*YALI0B20196g*	fatty acid elongase	Strong	$2.77x10^7$
	P_{YBR159}	*YALI0A06787g*	17beta-estradiol 17-dehydrogenase	Medium	$8.50x10^6$
	P_{PHS1}	*YALI0F11935g*	3-hydroxyacyl-CoA dehydratase	Weak	$2.87x10^6$
	P_{TER}	*YALI0A04983g*	enoyl-CoA reductase	Medium	$7.83x10^6$
	P_{ECH2}	*YALI0B10406g*	enoyl-CoA hydratase	Weak	$2.03x10^6$
	P_{OLE1}	*YALI0C05951g*	stearoyl-CoA desaturas	Medium	$1.00x10^7$
	P_{FAD2}	*YALI0B10153g*	omega-6 fatty acid desaturase	Medium	$6.77x10^6$
	P_{GPD1}	*YALI0B02948g*	glycerol-3-phosphate dehydrogenase	Medium	$5.23x10^6$
	P_{SCT1}	*YALI0C00209g*	dihydroxyacetone phosphate acyltransferase	Medium	$7.86x10^6$

Pathway	Name	Gene	Enzyme	Strength	Value
The fatty acids degradation	P_{ERG10}	*YALI0B08536g*	acetyl-CoA acetyltransferase	Medium	$4.95x10^6$
	P_{POT1}	*YALI0E18568g*	acetyl-CoA acyltransferase	Medium	$1.38x10^7$
	P_{POX1}	*YALI0E32835g*	acyl-CoA oxidase	Weak	$8.27x10^5$
	P_{POX2}	*YALI0F10857g*	acyl-CoA oxidase	Weak	$3.59x10^6$
	P_{POX4}	*YALI0E27654g*	acyl-CoA oxidase	Weak	$1.53x10^6$
	P_{POX6}	*YALI0C23859g*	acyl-CoA oxidase	Weak	$7.71x10^5$
	P_{POX3}	*YALI0C24750g*	acyl-CoA oxidase	Weak	$2.04x10^5$
	P_{ICL1}	*YALI0C16885g*	isocitrate lyase	Medium	$1.44x10^7$
	P_{YAT1}	*YALI0F21197g*	carnitine acetyltransferase	Weak	$4.05x10^6$

Noticeably, acetyl-CoA conversion to malonyl-CoA is a pivotal step in fatty acids synthesis, catalyzed by acetyl-CoA carboxylase ACC1[29]. However, our results found that the transcriptional expression level of P_{ACC1}, transcribing enolase ACC1, is relatively low, only $5.73x10^5$, which is 1.33% of the P_{TEF} promoter, suggesting acetyl-CoA carboxylase ACC1 is a rate-limiting step. Therefore, overexpression of acetyl-CoA carboxylase ACC1 could effectively increase the production of the malonyl-CoA derivatives[2, 8]. Specifically, the promoter of fatty acid elongase ELO1, P_{ELO1}, displayed the highest strength, reaching $2.77x10^7$, 64.41% of the P_{TEF} promoter. Moreover, we found that the transcript level of FAS1 (fatty acid synthase 1) was significantly lower than that of FAS2 (fatty acid synthase 2) during the whole fermentation process. Mainly, the transcriptional activities of desaturases, including P_{OLE1} (transcribing stearoyl-CoA desaturase OLE1) and P_{FAD2} (transcribing omega-6 fatty acid desaturase FAD2), also have been investigated. As shown in Fig. 3, P_{OLE1} and P_{FAD2} are both medium-strength promoters, but the activity of P_{OLE1} is dramatically higher. These results may be a guideline for directing the engineering of *Y. lipolytica* to produce unsaturated and polyunsaturated fatty acids.

Fatty acids degradation

In *Y. lipolytica*, lipogenesis involves the dynamic balance of fatty acid biosynthesis and degradation[18, 29]. Specifically, accumulated fatty acids would be degraded to maintain cellular metabolism by β-oxidation when carbon substrates are depleted[1, 30]. For example, two acetyl-CoA generated from β-oxidation are converted to C4 dicarboxylates (malate, succinate) for replenishing TCA intermediates by the glyoxylate shunt pathway in peroxisome[31]. Besides, Dulermo et al. demonstrated that inactivation of genes *POX1-6* in β-oxidation could improve fatty acids production, increasing to 65–75% of the dry

cell weight[32]. Therefore, β-oxidation is a vital branch of fatty acids metabolism that cannot be ignored in *Y. lipolytica*. At that point, we surveyed 9 promoters (Table 1).

Interestingly, our results showed that five promoters of acyl-CoA oxidase (POX) are all weak promoters, and their strengths ranged from $7.71x10^5$ to $3.59x10^6$ (Fig. 3). Notably, it has been demonstrated that the promoter P_{POX2} is induced by fatty acids, and P_{POX1} and P_{POX5} are induced by alkanes[33]. Specifically, the core sequences of a promoter in fungi are about 200–300 bp[18]. Nevertheless, we truncated 1500 bp sequences before ATG site of the desired gene as the corresponding promoter, suggesting that promoters obtained in this study should contain regulatory sequences of transcription factors. Therefore, it is believable that promoters P_{POX1} (*YALI0E32835g*), P_{POX2} (*YALI0F10857g*), and P_{POX5} (*YALI0C23859g*) still retain the inducible properties and, as a result, show a low activity under the condition without any additional inducers. Unexpectedly, the promoter P_{ERG10} (*YALI0B08536g*, transcribing stearoyl-CoA desaturase OLE1) and P_{POT1} (*YALI0E18568g*, transcribing stearoyl-CoA desaturase OLE2) displayed the high transcriptional activities with the strength of $4.95x10^6$ and $1.38x10^7$, respectively. In addition, the promoter P_{YAT1} (*YALI0F21197g*) transcribes carnitine acetyltransferase YAT1, and its strength reached $1.44x10^7$. The carnitine acetyltransferase YAT1 participates in the carnitine shuttle that transports the peroxisomal acetyl-CoA into mitochondria[34, 35]. The high strength of P_{YAT1} suggests that the carnitine shuttle is active in *Y. lipolytica*.

Other carbon metabolism

Moreover, we also analyzed several promoters in gluconeogenesis and other carbon utilization pathways, including P_{FBP1} (*YALI0A15972g*), P_{PGM2} (*YALI0E02090g*), P_{SOU1} (*YALI0B16192g*), and P_{MnDH2} (*YALI0D18964g*). We found that P_{FBP1} is a medium-strength promoter with the strength of $5.80x10^6$ and promoters, namely P_{PGM2} and P_{SOU1} are weak, while P_{MnDH2} is the strongest promoter in this study, 1.6-fold of the strength of the P_{TEF} promoter, reaching $6.87x10^7$.

Nitrogen metabolism

Nitrogen metabolism and its regulatory pathways play an essential role in the synthesis and catabolism of amino acids, proteins, and other nitrogen-containing substances, impacting the overall cellular metabolism[36]. Here, we investigated 14 promoters (Table 2), including 1 strong promoter that is P_{AAT1}, 7 medium-strength promoters that include P_{AAT2}, P_{AR08}, P_{AR09}, P_{HPD}, P_{UGA2}, P_{LEU2}, and P_{HPD1}, and 6 weak promoters that include P_{ALT1}, P_{HIS5}, P_{GAD1}, P_{EHD3}, P_{GLT1}, and P_{GLN1}, revealing the complicated regulation of the nitrogen metabolism. Notably, studies have shown that nitrogen metabolism in yeast mainly starts from glutamate and its derivative glutamine[37]. Specifically, glutamate could be converted from α-ketoglutarate, a metabolite in the TCA cycle, by glutamate synthase GLT1, glutamine synthetase GLN1, alanine transaminase ATL1, and cytoplasmic aspartate aminotransferase AAT1, and mitochondrial aspartate aminotransferase AAT2 in *Y. lipolytica*, which serves as a bridge linking the carbon and nitrogen metabolism. Our results (Fig. 4) showed that the strength of promoter P_{AAT1}, P_{AAT2}, P_{GLT1}, P_{GLN1},

and P_{ATL1} are 2.50x10^7, 1.45x10^7, 2.23x10^6, 1.51x10^6, and 3.88x10^6, respectively, which are 58.10%, 33.69%, 5.20%, 3.52%, and 9.01% of the P_{TEF} promoter. Generally, the muscular strength of P_{AAT1} and P_{AAT2} indicates that the synthetic metabolism of glutamate is mainly catalyzed by aspartate aminotransferase.

Table 2
The characterized promoters in the nitrogen metabolism

Pathway	Name	Gene	Enzyme	Strength	Value
Nitrogen metabolism	P_{AAT2}	*YALI0B02178g*	aspartate aminotransferase	Medium	1.45x10^7
	P_{AAT1}	*YALI0F29337g*	aspartate aminotransferase	Strong	2.50x10^7
	P_{UGA2}	*YALI0F26191g*	succinate-semialdehyde dehydrogenase	Medium	7.63x10^6
	P_{LEU2}	*YALI0C00407g*	3-isopropylmalate dehydrogenase	Medium	1.76x10^7
	P_{HPD1}	*YALI0F02607g*	3-hydroxyisobutyrate	Medium	4.93x10^6
	PE_{HD3}	*YALI0D06215g*	3-hydroxyisobutyryl-CoA hydrolase	Weak	1.79x10^6
	P_{GLT1}	*YALI0B19998g*	glutamate synthase	Weak	2.24x10^6
	P_{GLN1}	*YALI0D13024g*	glutamine synthetase	Weak	1.51x10^6
	P_{GAD1}	*YALI0C16753g*	glutamate decarboxylase	Weak	1.39x10^6
	P_{ALT1}	*YALI0D06325g*	alanine transaminase	Weak	3.88x10^6
	P_{AR08}	*YALI0E20977g*	aromatic amino acid aminotransferase	Medium	6.69x10^6
	P_{AR09}	*YALI0C05258g*	aromatic amino acid aminotransferase	Medium	6.03x10^6
	P_{HPD}	*YALI0B21846g*	4-hydroxyphenylpyruvate dioxygenase	Medium	2.03x10^7
	P_{HIS5}	*YALI0E01254g*	histidinol-phosphate aminotransferase	Weak	2.27x10^6

Moreover, aromatic amino acids can be used to synthesize several high-value compounds, such as p-coumaric acid, violacein, and flavonoids[9]. The transcriptional analysis showed that there were 3 medium-strength promoters, namely P_{AR08}, P_{AR09}, and P_{HPD}, and 1 weak promoter, namely P_{HIS5}, in the aromatic amino acid derivatives metabolism. The transcriptional activities of P_{AR08}, P_{AR09}, P_{HPD}, and P_{HIS5} are 6.69x10^6, 6.03x10^6, 2.03x10^7, and 2.27x10^6, respectively.

Other metabolisms

Apart from the carbon and nitrogen metabolism, several promoters of carriers, ribosomes, signaling proteins, and unknown-function proteins also have been analyzed. As shown in Fig. 4, the promoter P_{RSM7} (*YALI0D08470g*) transcribes ribosomal protein, which is a strong promoter with the strength of $2.50x10^7$, 58.18% of the P_{TEF} promoter. The signaling proteins promoters P_{SLY1} (*YALI0D20416g*), P_{MDR1} (*YALI0A18700g*), and P_{ARP4} (*YALI0E18986g*) have the strength of $7.22x10^6$, $2.23x10^7$, and $2.54x10^7$, respectively, which are 16.79%, 58.18%, and 59.09%. In addition, unknown-function proteins promoters P_{2034} (*YALI0C12034g*) and P_{8272} (*YALI0D08272g*) are strong promoters with the strength of $4.77x10^7$ (110.87% of the P_{TEF} promoter) and $2.29x10^7$ (55.50% of the P_{TEF} promoter), while P_{27533} (*YALI0F27533g*) is a medium-strength promoter with the strength of $6.45x10^7$ (14.99% of the P_{TEF} promoter). Particularly, the promoter P_{PHO89} (*YALI0E23859g*) is the weakest found in this study, with a 0.06% strength of the P_{TEF} promoter, which is responsible for transcribing sodium-dependent phosphate transporter.

Discussion

In this study, 81 endogenous promoters in *Y. lipolytica* were systematically investigated and analyzed. For obtaining the endogenous promoters, we truncated the 1500 bp upstream sequences before the ATG site of the desired gene. Specifically, it has been reported that the core sequences of a promoter in fungi are about 200-300bp. As a result, our obtained promoters in this study should contain the binding sites of the transcription factors, thus can accurately reflect some properties of these endogenous promoters. However, *Y. lipolytica* could generate severe fluorescence background, which interferes with the reliabilities of conventional reporter systems. Specifically, a luciferase reporter system has been developed in *Y. lipolytica*, which is stable, efficient, and instant[19]. Herein, we chose this luciferase reporter system for the promoter analysis.

Next, to construct the reporter system plasmids pYLXP'-P_{xx}-*Nluc*, we inserted the promoter sequences into the chassis plasmid pYLXP'-*Nluc*, and obtained 82 recombinant promoter plasmids, including the P_{TEF} promoter. Further, constructed plasmids were transformed into po1g for the promoter analysis. Specifically, we obtained 15 strong promoters, 41 medium strength promoters and 25 weak promoters. Among them, the most potent promoter is P_{MnDH2} with a 1.60-fold strength of the P_{TEF} promoter, reaching $6.87x10^7$, which is responsible for transcribing sorbose reductase, catalyzing the reaction of sorbose to glucitol. Furthermore, the weakest promoter is P_{PHO89} (*YALI0E23859g*), with a 0.06% strength of the P_{TEF} promoter, which is responsible for transcribing sodium-dependent phosphate transporter. These results suggest that we obtained an endogenous promoter library with a strength spanning from 0.06% to 1.60-fold of the P_{TEF} promoter. Although we only found several endogenous promoters with higher strength than P_{TEF} in this study, gene expression levels do not blindly pursue high strength of the promoter.

We explored the relationship between the promoter activity and time (Fig. 5), and found that 88.9% promoters had the highest intensity in the logarithmic growth phase (before 36h). And, 9.9% promoters had the highest intensity in the stationary phase (48–96 h), while only one promoter (P_{PGM2}, *YALI0E02090g*) reached the highest transcriptional activity at 72h. In addition, we also found that the promoter strength is different in the specific metabolic pathways. For example, promoters in glycolysis are generally more robust. In conclusion, the promoter library we constructed is significant for enriching genetic expression elements for *Y. lipolytica*, and can be applied to construct microbial cell factories for the biomanufacturing applications.

Materials And Methods

Strains, plasmid, primers, and chemicals

This study lists all stains, recombinant plasmids, and primers in Supplementary Table 1 and 2, respectively. Herein, strain po1g[38] was chosen as the starting strain. Chemicals used in this study, including glucose, $(NH_4)_2SO_4$, and YNB (yeast nitrogen base without $(NH_4)_2SO_4$ and amino acids), were all purchased from Sangon Biotech Co., Ltd (Shanghai, China). The Nano-Glo® Luciferase Assay System kit was purchased from Promega (Catalog: #N1120), and CSM-Leu powder was purchased from Sunrise Science Products (Catalog: #1001 − 100).

Construction of the luciferase reporter vectors

The reporter vectors were constructed based on the plasmid pYLXP', and NanoLuc luciferase is encoded by the gene *Nluc*[19]. Firstly, the chassis plasmid pYLXP'-*Nluc* was obtained by the Gibson Assembly method, using the gene *Nluc* fragment (PCR-amplified by primers Nluc-F and Nluc-R from pYLXP'-P_{TEF}-*Nluc*) and linearized pYLXP' (digested by *SnaBI* and *KpnI*). Then, plasmid pYLXP'-*Nluc* was further digested by *SnaBI* and *AvrII*, giving linearized pYLXP'-*Nluc*. Next, the promoter sequences of P_{xx} were obtained by PCR-amplified from the genome of *Y. lipolytica* using appropriate primers. Finally, the promoter sequences and linearized pYLXP'-*Nluc* were assembled to reporter plasmids pYLXP'-P_{xx}-*Nluc* by the Gibson Assembly method. The constructed plasmids were all sequenced by Sangon Biotech Co., Ltd (Shanghai, China).

Yeast transformation by the lithium acetate method

The standard protocol of the lithium acetate yeast transformation has been described in the previous report[3]. Briefly, cells were harvested from 0.5 ml culture solution at 24 h using the YPD medium by the shaking tube. Then, cells were washed twice using the phosphate buffer (PBS, 100 mM, pH 7.0) and resuspended by the transformation solution (105 uL), containing the lithium acetate (2M, 5 uL), PEG4000 (50%, 90 uL), boiled single strand DNA (salmon sperm, denatured, 5 uL), and reporter plasmids (5 uL). Next, the mixture was incubated at 39°C for one hour, which needed to be vortexed for 15 seconds every 15 minutes. Finally, the mixture was spread on the CMS-leu selected plates. YPD medium used in this study included glucose 20 g/L, peptone 20 g/L, and yeast extract 10 g/L.

Shaking flask cultivations

For this, seed culture was carried out in the seed culture medium (2 mL) at 30 ^{o}C and 250 r.p.m for 48 h, using the shaking tube. Then, seed culture (0.8 mL) was inoculated into the CSM medium (C/N = 80, 30 mL) in the 250 mL flask and grown at 30 ^{o}C and 250 r.p.m for 120 h. During the process of fermentation, 1 ml culture solution was sampled every 12 h for luciferase and OD_{600} measurements. The seed culture medium contains yeast nitrogen base without ammonium sulfate (YNB) 1.7 g/L, glucose 20.0 g/L, $(NH_4)_2SO_4$ 5.0 g/L, and CSM-Leu 0.74 g/L. Moreover, the CSM medium (C/N = 80) contains YNB 1.7 g/L, glucose 40.0 g/L, $(NH_4)_2SO_4$ 5.0 g/L, and CSM-Leu 0.74 g/L.

Quantification of cell densities and the promoter strength

Cell densities of *Y. lipolytica* were monitored by measuring the optical density at 600 nm (OD_{600}). The promoter strength was determined by performing the luciferase whole-cell assay analysis. In detail, 0.5 ml culture solution was centrifuged at 8,000 r.p.m for 3 min to collect the cell pellet. Then, the collected cell pellets were washed twice using the phosphate buffer (PBS, 100 mM, pH 7.0), and resuspended by the same buffer (1 ml). It should be noted that OD_{600} of cell pellet suspension solution needed to be measured and recorded. Next, the reaction mixture of the luciferase whole-cell assay was prepared for the luciferase activity assay by the microplate system (following the protocol of Nano-Glo® Luciferase Assay System kit), which contained the luciferase buffer 100 ul, substrate 2ul, cell pellet suspension solution 10 ul, and sterile water 88 ul. As a result, the promoter strength was obtained by dividing the luciferase activity data by the recorded OD_{600} of the cell pellet suspension solution.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YG and XS conceived the topic. CW performed genetic engineering and fermentation experiments with input from XL, ML, YL, LH and JZ. YG and CW wrote the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

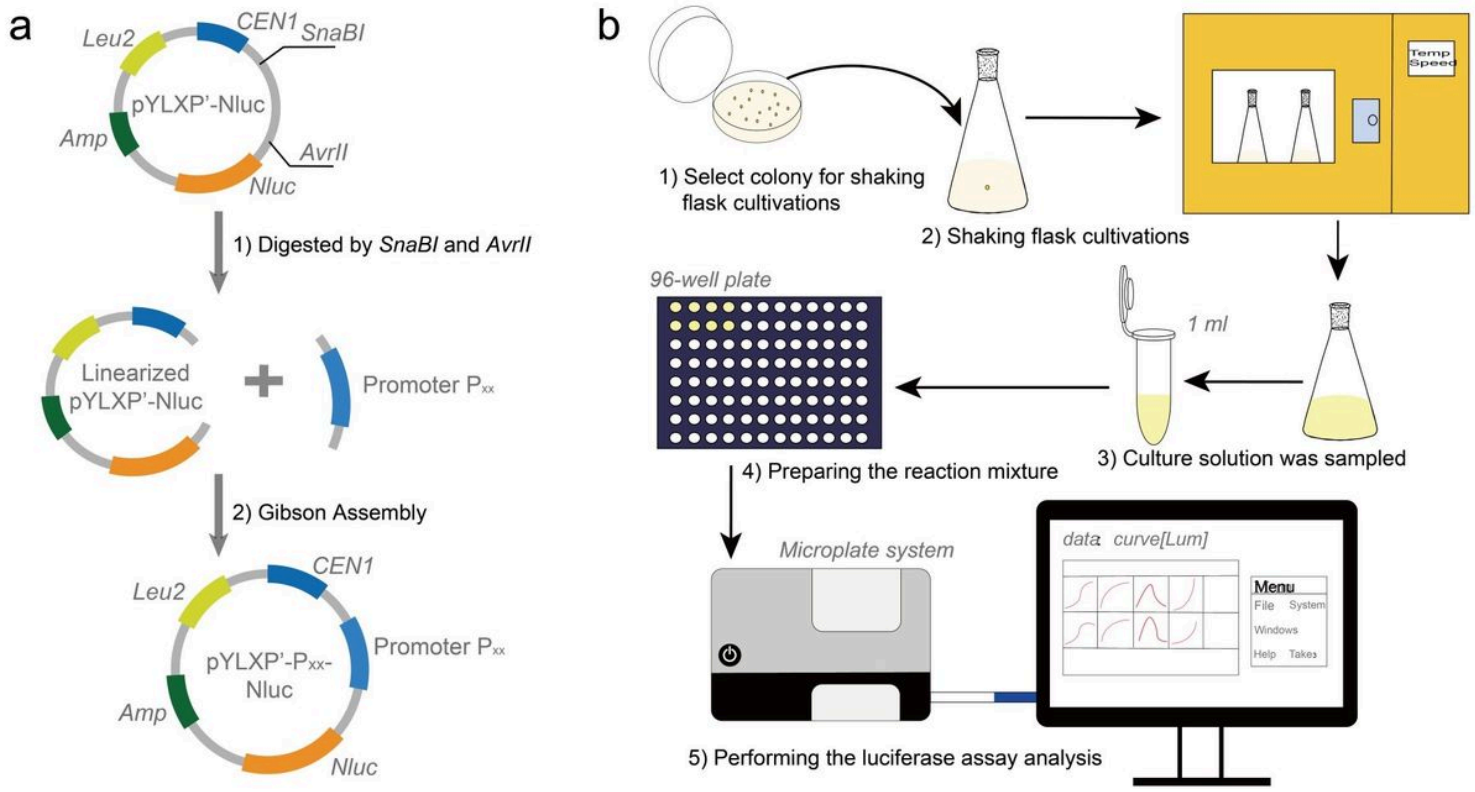

Figure 1

The process of constructing the reporter vectors and characterized the promoter strength. (a) Construction of the luciferase reporter vectors for characterizing the endogenous promoters; (b) Shaking flask cultivations for quantification of cell densities and the promoter strength.

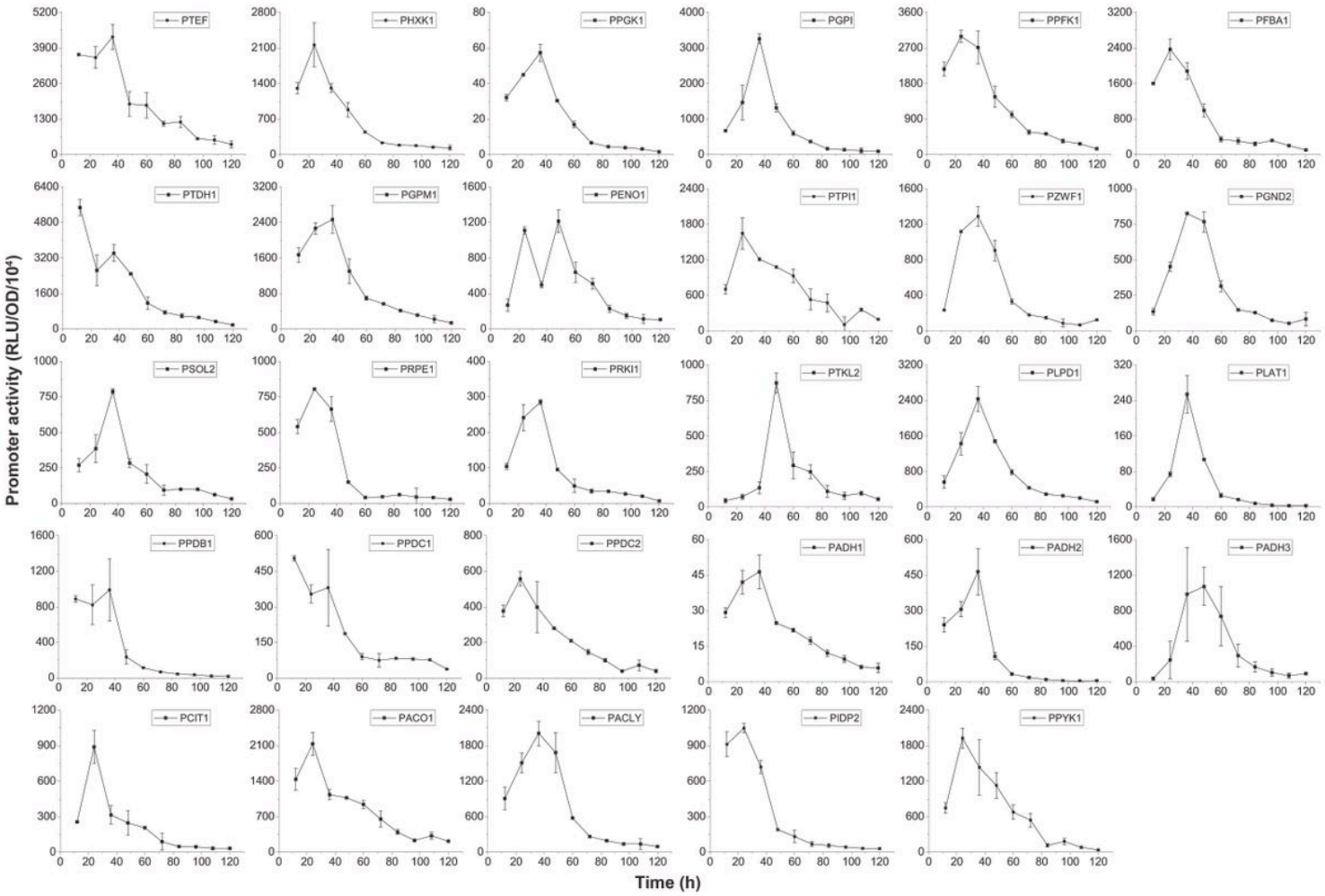

Figure 2

The activities of the characterized promoters in the glycolysis pathway, pentose phosphate pathway, pyruvate metabolism and tricarboxylic acid cycle.

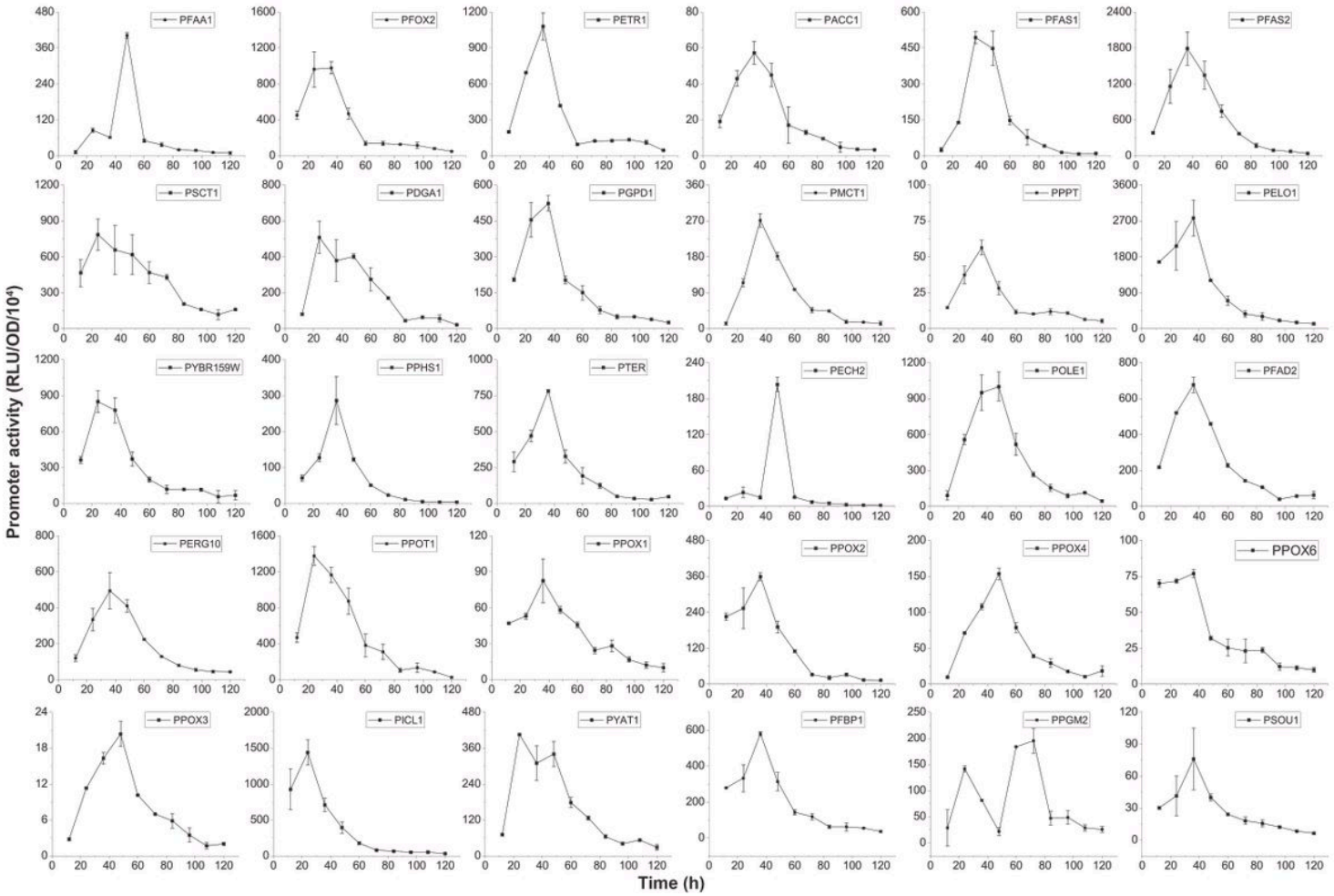

Figure 3

The activities of the characterized promoters in the lipogenesis and other carbon metabolism.

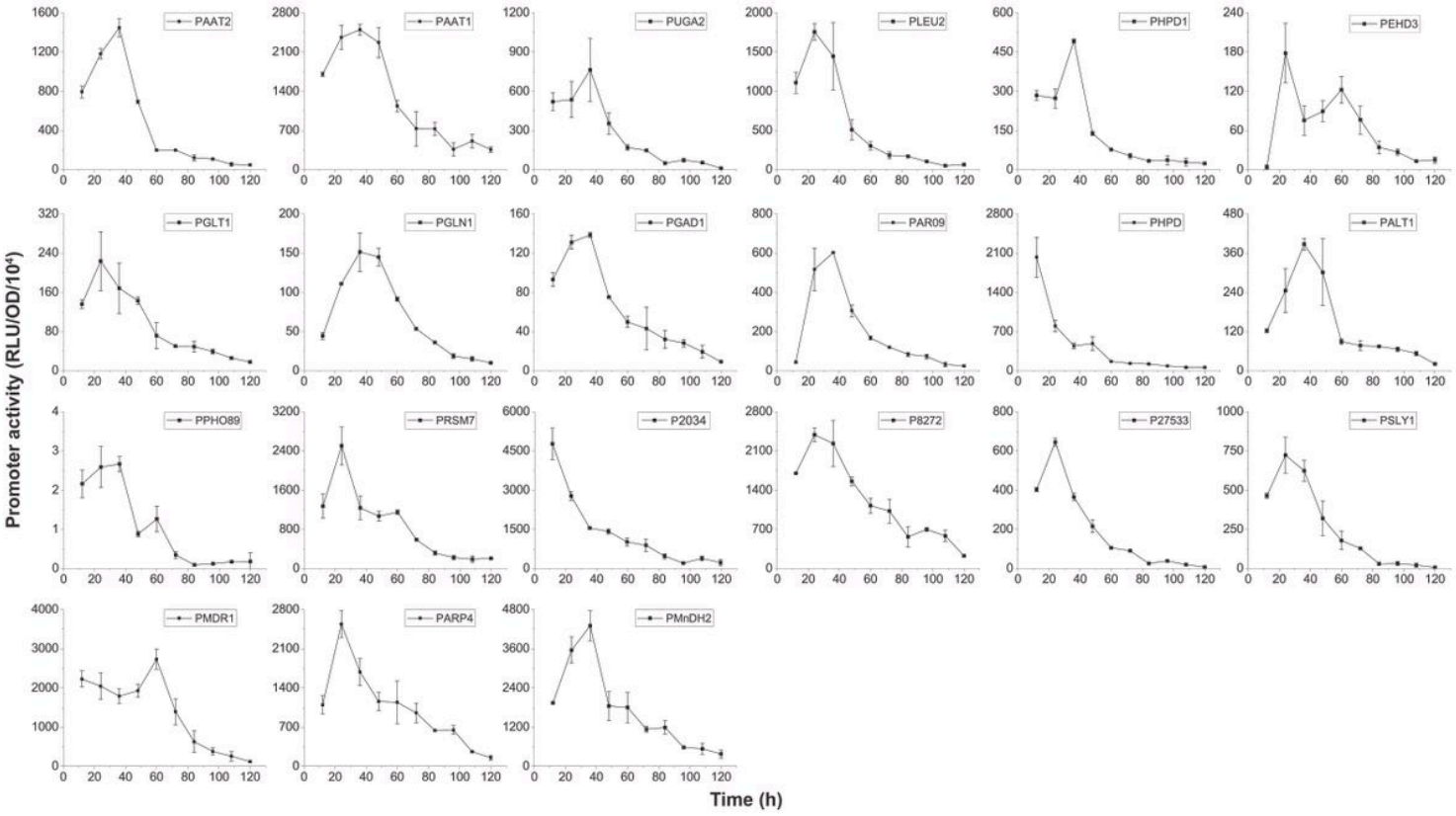

Figure 4

The activities of the characterized promoters in the nitrogen metabolism and other metabolisms.

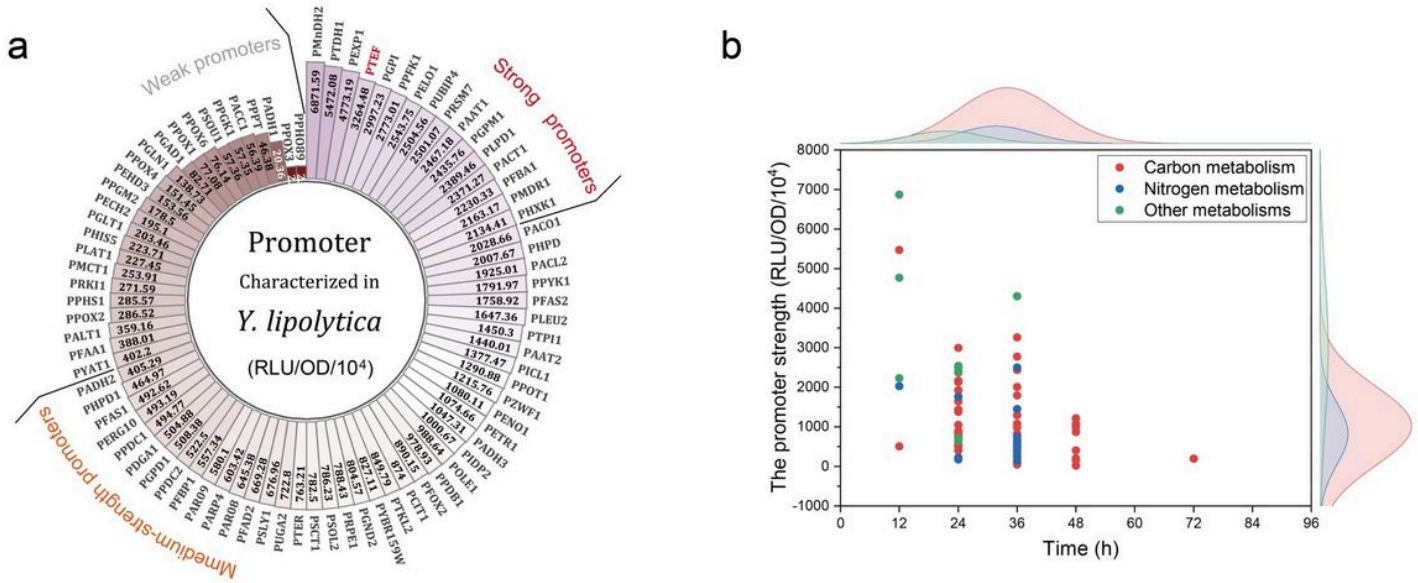

Figure 5

Comparison of the endogenous promoters in *Y. lipolytica*. (a) Evaluation of the promoter strength; (b) Evaluation of the relationship between the promoter activity and time.

Supplementary Files

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