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Original Research Article

Promoter engineering for enhanced 3-hydroxypropionic acid production in *Pichia pastoris*

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ABSTRACT

Enabling tools are essential for facilitating the methanol bioconversion in *Pichia pastoris*. However, there is still a relative lack of promoters that can stably express high levels without being affected by the carbon source, which hinders the construction and modification of cell factories containing long metabolic pathways. This study mapped a gene expression intensity library of central metabolic pathways in *P. pastoris* under methanol and glucose conditions. Through modification of the upstream sequences of promoters, an artificial promoter P_{S2} was developed with a strong intensity up to 90 % of P_{GAP}. By using this promoter, we successfully constructed a hybrid pathway that integrates the β-alanine and malonyl-CoA pathways for the production of 3-hydroxypropionic acid. Further combining rational metabolic engineering strategies, such as optimizing gene copy numbers and blocking byproduct synthesis pathways, the engineered strains CHP9 and CHP20 achieved 3-HP titers of 23 g/L and 22 g/L by using methanol as the sole carbon source. These results indicate that adaptive strength of promoters can facilitate efficient chemical biosynthesis in methanol bioconversion by mitigating glucose repression effects. This work preliminarily explored the expression patterns of genes in the central metabolic pathways of *P. pastoris*, identified and characterized the intensities of various endogenous promoters, and extended the enabling toolbox for *P. pastoris*. This result also lays a foundation for the construction of microbial cell factories and the industrial production of 3-HP via methanol bioconversion.

1. Introduction

Pichia pastoris (also known as *Komagataella phaffii*) is an unconventional methylotrophic yeast capable of using methanol as sole carbon and energy source. Owing to the characters of robustness, high-density growth [1] and tightly regulated methanol-inducible expression system, which making *P. pastoris* a widely used host for recombinant protein production [2,3]. *P. pastoris* is also recognized as Generally Recognized as Safe (GRAS) [4], which greatly expands its application as microbial cell factory platform. Recombinant protein expression typically involves in single gene modification. However, the production of complex chemicals usually required the reconstruction of metabolic pathways and regulating gene expressions. This process heavily relies on enabling tools, including the CRISPR-Cas9 gene-editing system and promoters expression intensity [5]. In *P. pastoris*, despite CRISPR-Cas9 gene-editing tools were successfully constructed, and the gene editing efficiency improved either by enhancing homologous recombination [6,7] or blocking non-homologous end joining process [8], the genetic elements such as promoters are still deficient in *P. pastoris*.

In metabolic engineering, efficient genetic tools facilitate the precise manipulation of bioconversion pathways. Promoter is core enabling element in gene expression systems, specifically binding RNA

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polymerase to drive the transcription of target genes, and level of gene expression [9,10]. Despite significant efforts have been made to explore the promoters in *P. pastoris* [11–15], the available endogenous promoters remain quite limited, making challenges for efficient regulating gene expression involved in metabolic pathways during methanol bioconversion. Currently, the most commonly used promoters in *P. pastoris* are the methanol-inducible strong promoters P_{AOX1} (alcohol oxidase I) [16] and P_{DAS2} (dihydroxyacetone synthase) [17]. A library of P_{AOX1} promoter variants with enhanced activity has been developed through targeted modification of the underlying transcription factors [18]. As well as the strong constitutive promoters P_{GAP} (Glyceraldehyde-3-phisphate dehydrogenase) [19] and P_{TEF1} (transcriptional elongation factor) [10], a promoter library containing a P_{GAP} promoter mutant with transcriptional activity about 48-fold greater than that of the wild-type was obtained through random mutation [20]. Additionally, inspired by natural bidirectional promoters, an artificial bidirectional promoter library has been developed as an efficient solution for gene co-expression [21]. However, the repeated use of the same promoter can lead to noticeable competition effects, reducing the transcriptional expression levels of target genes and causing cellular instability, such as homologous recombination and gene loss [13]. Common promoter elements in yeast including Upstream Activating Sequences (UAS), TATA box and core promoter [22]. Among them, UAS serves as the binding site for transcription factors and RNA polymerase II, and thus can be fine-tuned through rational modification [23–25]. In eukaryotes, the design of synthetic core promoters also has significantly enhanced the performance of synthetic variants [26]. Blazeck [27] demonstrated that by combining different UAS elements (UAS_{TEF1} and UAS1B) and arranged 12 UAS_{TEF1} in tandem, the strength of promoter can be enhanced by 3–4.5 folds, achieving nearly 7-fold higher than the strong constitutive promoter P_{TEF1}. An artificial promoter *YGP1v7* with high activity in acidic environment was obtained by replacing the core promoter region and inserting transcription factor binding sites in UAS [28]. Ye [29] constructed two hybrid growth phase-dependent promoters (Phy3 and Phy4) by tandem fusing UAS region, which had 2-fold increased activities compared to the wild-type promoters (Pro-Y13 and Pro-Y14). Therefore, exploring and modification of more promoters is an effective strategy to expand the genetic regulation elements for methanol bioconversion and cell factory construction in *P. pastoris*.

P. pastoris is now well developed to bioconvert methanol into varieties of chemicals, including 3-hydroxypropionic acid (3-HP) [23], fatty acids [30], fatty alcohols [31], polyketides [32], isoprenoids [33], and L-lactic acid [34], however, the titers are generally lower than those achieved through sugar-based fermentation. 3-HP is one of the 12 key platform chemicals [35] widely used in the chemical, pharmaceutical, food and agricultural industries [36]. The biosynthetic pathways for 3-HP are diverse [37], and the malonyl-CoA reductase (MCR) pathway is particularly notable due to its fewer steps and the high availability of the precursor acetyl-CoA [38]. According to predictions from genomic metabolic models, the β-alanine pathway offers a higher theoretical yield when methanol or glycerol is used as the sole carbon source [37,39] (Supplementary Table S1). This indicates that the β-alanine pathway is a potential option for the economical production of 3-HP.

In this study, we first characterized the endogenous promoters of central metabolic pathway genes in *P. pastoris*, and identifying the medium-strength constitutive promoter P_{PYC2}. By using promoter prediction tools to assist the truncation of the upstream sequences, we obtained a variant promoter P_{S2} with double transcriptional activity compared to P_{PYC2}. We also constructed various hybrid promoters by linking different UAS regions to enhance activity. Subsequently, we established a hybrid pathway combining the β-alanine pathway and malonyl-CoA pathway, using promoters P_{S2} and P_{AOX1} to regulate the β-alanine-pyruvate transaminase genes (*BAPAT*) and 3-hydroxypropanoate dehydrogenase gene (*YDFG*) or malonyl-CoA reductase N-terminus (*MCRN*), respectively. The engineered strain CHP9 and CHP20 achieved 3-HP titers of 23 g/L and 22 g/L in bioreactors, with a yield of 0.13 g/g methanol. This study not only enriches the enabling toolbox for *P. pastoris* but also provides engineering strategy for construction of hybrid 3-HP biosynthetic pathways.

2. Results and discussion

2.1. Screening promoters related to central metabolism pathways in P. pastoris

In previous studies, we characterized the promoters of partial genes related to methanol metabolic pathways and expanded the enabling toolbox of *P. pastoris* [6]. However, methanol-induced promoters are often inhibited by glucose and other carbon sources, and there are still only a few strong constitute promoters such as P_{GAP} and P_{TEF1}, which are often used for gene expression during methanol metabolism. To identify more powerful promoters, we expanded the screening to several central metabolism pathway genes that are crucial for maintaining cellular activities. We screened 28 gene promoters from central carbon metabolism that have not yet reported promoter strength, mainly including genes from glycolysis (Embden-Meyerhof pathway, EMP), tricarboxylic acid cycle (TCA) and pentose phosphate pathway (PPP) (Fig. 1a, Table S2).

The key promoter regulatory elements are typically located >500 bp upstream of the start codon (ATG), with optimal promoter lengths ranging from 1000–1500 bp [40,41]. Consequently, the 1000 bp upstream regions of these genes were adopted as candidate promoters. We then used green enhanced fluorescent protein (eGFP) to evaluate promoter strength [42]. A series of different eGFP expression cassettes were integrated into the neutral site *PNSII-5* using the CRISPR-Cas9 gene editing system, and their expression levels were evaluated in the medium of glucose or methanol. The results showed that the promoter strength of most central metabolic pathway genes remained moderate and uniform during glucose culture. It was speculated that central metabolic pathways must rapidly respond to energy and nutrient changes. The promoters with basal-level expression can be rapidly modulated by transcription factors or cofactors to maintain metabolic network balance. The relative strong promoters were come from some genes that involve special regulatory processes. For example, the expression levels of enolase (*ENO1*), phosphoglycerate kinase (*PGK1*) and 6-phosphogluconate dehydrogenase (*GND2*) reached to 83 %, 54 % and 35 % of the P_{TEF1} promoter at 24 h, respectively (Fig. 1b), and the growth of these strains were not affected (Fig. S1a–d).

In methanol medium, the expression level of most promoters were relatively uniform and the expression intensity were basically the same as that in glucose, which proved that these promoters belonged to constitutive promoters (Fig. 1c and Fig. S1e–h). Besides, the promoter P_{PYC2} (pyruvate carboxylase gene) showed the highest expression level, reaching to 75 % of P_{TEF1} at 72 h, and the expression intensity of P_{GND2} reaching to 57 % of P_{TEF1} at 72 h, which significantly higher than that of other promoters. The intensity of P_{PYC2} and P_{GND2} promoters were moderate expressed in glucose while high expressed in methanol, this can effectively avoid the inhibition of glucose on methanol medium and improved the catalytic processes, and would have potential application in the chemical biosynthesis from methanol.

2.2. Promoter engineering enhanced expression level

The construction of cell factories usually requires high expression levels of exogenous genes, but the weak expression of promoter P_{PYC2} requires further modification to enhance its expression level. Basically, promoter, consists of a core promoter and Upstream Activating Sequence (UAS) region, is the binding site for transcription factors and RNA polymerase II, and the UAS region is the key for improving promoter activity [23,43]. Therefore, we attempted to further enhance the activity of P_{PYC2} promoter by identifying the core promoter and modifying the UAS sequence. The core promoter region of P_{PYC2} promoter was identified using an online Neural Network Promoter Prediction tool

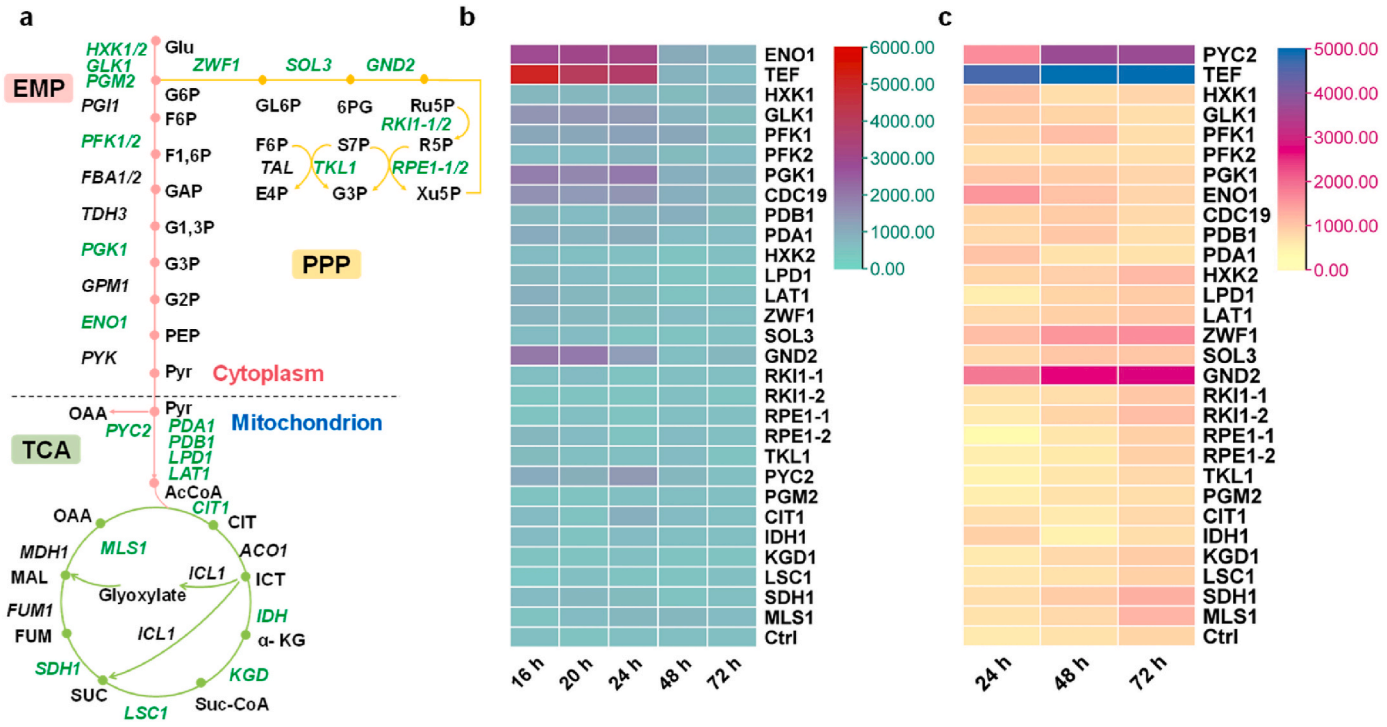

Fig. 1. Promoters screening of the central carbon metabolism pathways of *P. pastoris*. **(a)** The main metabolic pathways in *P. pastoris,* including EMP (Glycolysis), TCA (citric acid cycle) and PPP (pentose phosphate pathway). Green genes represent the genes corresponding to the promoter screened in this work. **(b)** Thermogram characterization of promoters intensity based on the central carbon metabolism pathways of *P. pastoris* in 20 g/L glucose and **(c)** in 10 g/L methanol. Genes abbreviation: *HXK1/2*, Hexokinase isoenzyme. *GLK1*, Glucokinase. *PGM2*, Phosphoribomutase. *PFK1*/2, 6-phosphofructokinase. *PGK1*, 3-Phosphoglycerate kinase. *ENO1*, Enolase I. *CDC19*, Pyruvate kinase. *PDA1*, E1 alpha subunit of the pyruvate dehydrogenase (PDH) complex. *PDB1*, E1 beta subunit of the pyruvate dehydrogenase (PDH) complex. *LPD1*, dihydrolipoyl dehydrogenase. *LAT1*, Dihydrolipoamide acetyltransferase component. *ZWF1*, Glucose-6-phosphate 1-dehydrogenase. *SOL3*, Phosphogluconate Dehydrogenase. *GND2*, 6-phosphogluconate dehydrogenase. *RKI1-1/2*, Ribose-5-phosphate ketol-isomerase. *RPE1-1/2*, D-ribulose-5-phosphate 3-epimerase. *TKL1*, Transketolase. *PYC2*, pyruvate carboxylase. *CIT1*, Citrate synthase. *IDH1*, isocitrate dehydrogenase. *KGD1*, Mitochondrial alpha-ketoglutarate dehydrogenase complex component. *LSC1*, succinyl-CoA synthetase. *SDH1*, succinate dehydrogenase. *MLS1*, Malate synthase.

(BDGP, fruitfly.org) with a length of about 50 bp (−898 ~ −849/−920 ~ −871). Subsequently, according to the transcription factor binding sites predicted by the online tool (Alibaba2, gene-regulation.com), the UAS sequence of the P_{PYC2} promoter was reasonably divided into four fragments [29] (Fig. 2a). We designed four P_{PYC2} promoter variants, P_{S1}–P_{S4}, containing different length of UAS sequences, and characterized the performance of these modified promoters by fluorescence intensity of eGFP expression. The results showed that the eGFP fluorescence expressed by P_{S1} was almost unable to detect, indicating that it only contained the core promoter region and lack of effective upstream activation sequences (Fig. 2b and Fig. S2a). In addition, the cell growth were almost unaffected (Fig. S2b–c). The strength of promoter P_{S2} was significantly increased by 197 % compared to the unmodified P_{PYC2} and reached almost the same level as P_{GAP} (90 %) and 64 % higher than P_{TEF1}. However, the expression levels of P_{S3} and P_{S4} showed that, further prolong the UAS sequence leading to a decrease in eGFP expression, suggesting that the upstream sequence of P_{S2} may contain some negative regulatory elements. Based on the above results, we preliminarily define the sequence extending from P_{S1} to P_{S2} as UAS_{PYC2}.

A transcription amplifier can be formed by increasing the copy numbers of UAS, and the activity of the modified promoter can be increased exponentially [43]. Here, we attempted to further enhance P_{S2} expression by adding UAS_{PYC2} or UAS_{AOX1} (UAS sequences from P_{AOX1}, -638∼-510 are *cis*-acting elements) [41] at the 5′ end of P_{S2}. The UAS_{AOX1} and UAS_{PYC2} (UAS_{AOX1}-UAS_{PYC2}) were combined in tandem to create hybrid promoter M1. Additionally, two UAS_{PYC2} sequences were repeated in tandem [44] ($2UAS_{PYC2}$) to form hybrid promoter Y1 by double enzyme digestion (Fig. 2c). However, the activity of the hybrid promoters were almost identical to that of P_{PYC2} (Fig. 2d and Fig. S2d) when fluorescence intensity was characterized using eGFP. Given that the regulatory mechanisms of core promoters in yeast systems can significantly influence promoter activity [23], it was speculated that the lack of synergy between the UAS and core promoters from different promoters may hindered gene expression regulation. Consequently, the promoter P_{S2} obtained from this truncation was retained for metabolic engineering.

2.3. Promoter P_{S2} improved the biosynthesis of 3-HP from methanol

3-HP is a promising chemical that can be synthesized through MCR pathway and β-alanine pathway in yeast [45]. In previous study, we obtained a strain with high 3-HP production by using methanol as carbon source through the MCR pathway [46]. Therefore, we focused on evaluating the biosynthesis of 3-HP via the β-alanine pathway (Fig. 3a). β-alanine as a crucial intermediate during 3-HP biosynthesis, we firstly attempted to enhance β-alanine biosynthesis (strain PCS2) by heterologous expression of the aspartate dehydrogenase gene *BaAspDH* from *Brucella anthropic* (genbank: ABS17096.1) and *BsADC* from *Bacillus subtilis* (NCBI Reference Sequence: WP_161619406.1) [47,48]. Considering that natural β-alanine synthesis is not efficient and *ADC* maybe a rate-limiting step, we added an additional copy of *BsADC* gene on chromosome to obtain the strain PCS3. The accumulation of 3-HP were not detected in both wild-type strain (G4RCH) and PCS2, but it was surprising that the 3-HP titer of PCS3 strain reached to 120 mg/L in 120 h (Fig. 3b and Table 1). These results indicated that all genes required for 3-HP biosynthesis by β-alanine pathway were naturally existed in *P. pastoris*, but metabolic flux was not sufficient to drive 3-HP

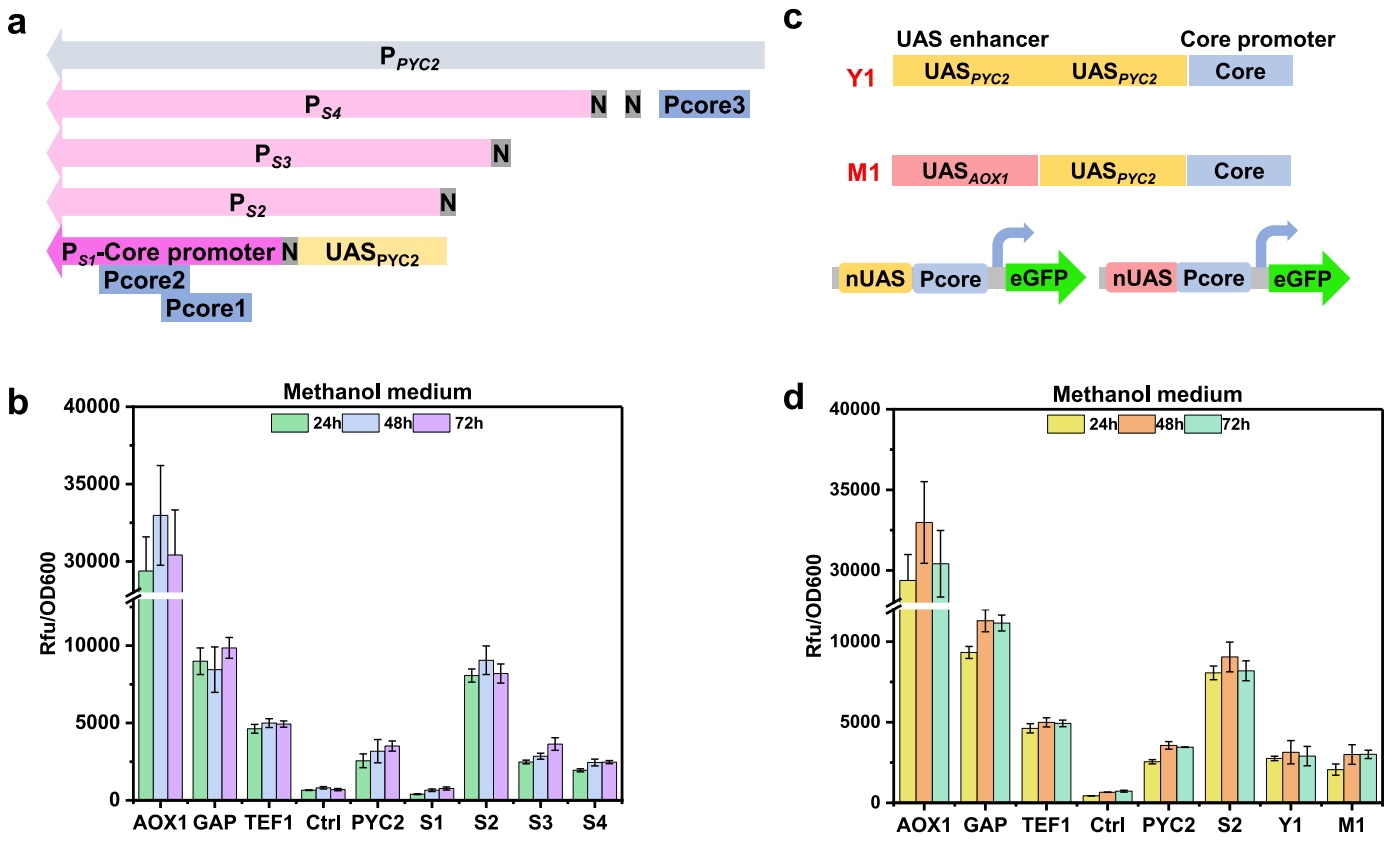

Fig. 2. Promoter engineering modification of P_{PYC2}. **(a)** The UAS region of P_{PYC2} was determined based on transcription factor binding sites and Neural Network Promoter Prediction by a stepwise truncation strategy. P_{S1}-P_{S4}: The P_{PYC2} promoter was truncated into four variants P_{S1}~P_{S4} of different lengths rationally. Pcore: The core promoter of prediction. N: The transcription factor non-binding sites of prediction. UAS: The core promoter of determination. **(b)** The UAS region was determined by the fluorescence intensity of the P_{PYC2} promoter variants in 10 g/L methanol minimal medium. **(c)** Construction of hybrid promoters $2UAS_{PYC2}$ and UAS_{AOX1}-UAS_{PYC2}. **(d)** Activity characterization of the hybrid promoters Y1 ($2UAS_{PYC2}$) and M1(UAS_{AOX1}-UAS_{PYC2}) strains. Error bars represent SD of triplicate samples.

biosynthesis. We then continued to expressed the third copy of *ADC* gene, and finally increasing 3-HP production by 17 %—141 mg/L (Fig. 3b). As a node compound in the metabolic network, pyruvate is also be a precursor for various compounds such as acetic acid, ethanol andlactic acid, and reducing metabolic flux toward 3-HP synthesis. Here, knocking out the aldehyde dehydrogenase gene *ALD4*, which is involved in acetic acid synthesis, significantly increased the production of 3-HP to 256 mg/L in strain PCS15. In addition, the production of the precursor β-alanine in these strains were also measured, and the results showed that with the accumulation of precursor β-alanine (Fig. 3b), the production of 3-HP also gradually increased. This indicated that enhancing the precursor supply is conducive to the synthesis of products.

β-alanine is catalyzed by a two-step enzyme reaction to produce 3-HP. First, the intermediate product malonate semialdehyde (MSA) is generated under the catalysis of β-alanine pyruvate aminotransferase. Then 3-hydroxypropionic dehydrogenase catalyzes the formation of 3-HP. To further enhance the synthesis of 3-HP, we expressed the β-alanine pyruvate aminotransferase gene *BcBAPAT* [37] from *Bacillus cereus* (GenBank: EEL86940) to construct PCS16 strain, and the 3-HP titer was sharply increased by 5.1 times to 1.3 g/L (Fig. 3c). The results verified that *P. pastoris* contains endogenous 3-hydroxy acid dehydrogenase. However, when the strain PCS23 was further expressing the 3-hydroxypropionic dehydrogenase *EcYDFG* [49] from *Escherichia coli* (NCBI: NP_416057.1), the production of 3-HP was significantly reduced (Fig. 3c). Due to the NADPH-dependence of 3-hydroxypropionic dehydrogenase, redox imbalance may occurred if using stronger promoters [50], thus reducing the production of PCS23 strain by 55 %. It has been reported that the use of moderate strength promoters, such as P_{PDC1} (pyruvate decarboxylase promoter) [51] and P_{POR1} (mitochondrial porin promoter) to drive the expression of *EcYDFG* were more contribute to the production of 3-HP [50]. Therefore, we used P_{PDC1} and P_{S2} promoters to express *EcYDFG* gene in strain PCS16 to generate strains CHP11 and CHP20, respectively. Shake flask fermentation showed that CHP11 and CHP20 produced 1.5 g/L 3-HP in 20 g/L methanol culture, which was 12.3 % higher than that of PCS16 strain (Fig. 3c). The results also demonstrated that P_{S2} promoter was helpful for high level chemical production by moderately regulating gene expression.

During the synthesis of 3-HP, the intermediate product MSA is generated either through β-alanine pathway or MCR pathway [46]. Considering 3-hydroxypropanoate dehydrogenase may have bidirectionally catalytic activity, and its high expression is not conducive to the accumulation of 3-HP. Therefore, we reconstructed a hybrid pathway by separately expressing the *BcBAPAT* gene coupled with *EcYDFG* or *MCRN*, or through a fusion expression using the linking peptide GGGGS. EcYdfg or McrN catalyze the reduction of MSA to 3-HP in the β-alanine pathway and the MCR pathway, respectively. The synthesis of 3-HP is drived by the promoters P_{AOX1} and P_{S2}. The results showed that the 3-HP titer of CHP9 strain (*MCRN* at N-terminal) could reach to 1.5 g/L, which was 16.2 % higher than strain CHP8 (*MCRN* at C-terminal). Meanwhile, 3-HP production was 18.7 % higher in fusion expression than in separate expression, which further proved that fusion design could make full use of intracellular intermediate metabolites for 3-HP biosynthesis (Fig. 3d). Here, we also tried to replace the *MCRN* promoter with P_{S2}, and achieving 3-HP titer comparable to CHP9. These results suggested that

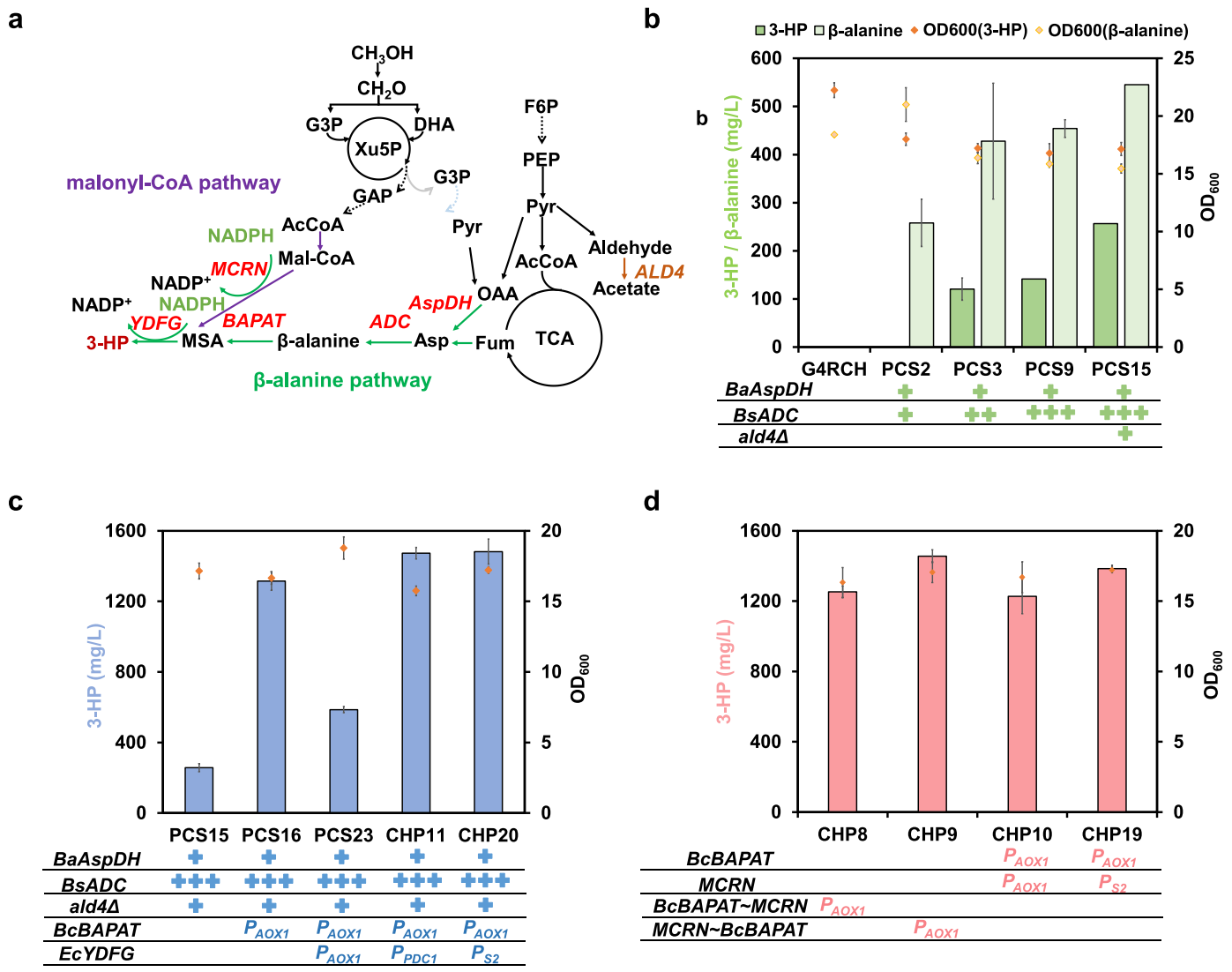

Fig. 3. Construction of cell factory for 3-HP production. **(a)** Schematic diagram of 3-HP biosynthesis through hybrid pathway including β-alanine and malonyl-CoA pathway in *P. pastoris*. The purple lines represent the malonyl-CoA pathway, the green lines represent the β-alanine pathway, the red genes were heterologously expressed, and the brown genes represent genes of knockout. **(b)** Construction of 3-HP synthetic strain. β-alanine and 3-HP production of precursor strains. **(c)** The biosynthesis of 3-HP were regulated by different promoters in β-alanine pathway. **(d)** The production of 3-HP biosynthesis were regulated by the P_{S2} and P_{AXO1} promoters through hybrid pathway. Error bars represent SD of triplicate samples.

the synthesis of 3-HP by MSA required a moderately strong promoter in β-alanine pathway and a strongly induced promoter in the malonyl-CoA pathway.

2.4. Fed-batch fermentation

We finally conducted fed-batch fermentation of strains CHP9 and CHP20 using methanol as sole carbon source. We found that, strains CHP9 and CHP20 produced 4.5 g/L and 4.6 g/L 3-HP, respectively, with a yield of about 0.12 g/g methanol after 112 h of fed-batch fermentation in 250 mL shake flask containing 50 mL minimum medium (Fig. 4a–b). In the process of microbial fermentation, suitable dissolved oxygen is very important for the product synthesis [52], and the low production in the flask maybe caused by the insufficient dissolved oxygen, the regulation of pH and other reasons. We speculated that the strain had a more stringent demand for fermentation conditions when using methanol as carbon source, and it is challenging to provide sufficient dissolved oxygen and adjust the pH during the shake flask fermentation. We thus performed fed-batch fermentation in 1 L bioreactors. The fermentation process was divided into two stages, in the first stage, glycerol was used as carbon source for the accumulation of biomass. When the biomass reached to 35 g/L dry cell weight (DCW), methanol was added for the 3-HP production stage. Finally, the engineered strains CHP9 and CHP20 produced 23 g/L and 22 g/L 3-HP with the yields of 0.12 g/g methanol and 0.14 g/g methanol, respectively (Fig. 4c–d). In addition, strains CHP9 and CHP20 contained 2.6 g/L and 2.9 g/L intracellular 3-HP, corresponding to 12 % and 13 % of the total production, respectively (Fig. S3a–b). The results is comparable to the reported highest production of 3-HP produced by β-alanine pathway using methanol as carbon source [50,53]. It was confirmed that P_{S2} promoter and hybrid pathways can effectively promote the biosynthesis of 3-HP.

As an organic acid, the accumulation of 3-HP within cells may bring metabolic stress. Future efforts could focus on screening suitable carboxylic acid transport proteins [54,55] to enhance the export of 3-HP from inside to outside the cell, potentially increasing the capacity of cell factories to produce 3-HP. Additionally, studies have reported that overexpression of the transcription factors Mxr1 and Mit1 can strengthen methanol metabolism and quadruple L-lactic acid production [56]. This strategy may also be applicable to enhance the biosynthesis of 3-HP.

Table 1
Main strains used in this study.

Strain	Description	Source
G4RCH	*Mut*$^+$, *AOX1*, *AOX2*, *his4::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1})=*GS115*, *his4Δ::HIS4*, *PNSI-3::* (P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1})	This study
PCS3	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3})	This study
PCS9	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3})	This study
PCS15	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *ald4Δ*	This study
PCS16	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *ald4Δ*, *PNSIII-12::*(P_{AOX1}-*BcBAPAT*-T_{FBP1})	This study
PCS23	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *ald4Δ*, *PNSIII-12::*(P_{AOX1}-*BcBAPAT*-T_{FBP1}), *PNSIV-11::*(P_{AOX1}-*EcYDFG*-T_{FBP1})	This study
CHP9	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *ald4Δ*, *PNSIV-9::*(P_{AOX1}-*MCRN-GGGS-BcBAPAT*-T_{FBP1})	This study
CHP11	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *ald4Δ*, *PNSIII-12::*(P_{AOX1}-*BcBAPAT*-T_{FBP1}), *PNSIV-11::*(P_{PDC1}-*EcYDFG*-T_{FBP1})	This study
CHP20	*Mut*$^+$, *AOX1*, *AOX2*, *his4Δ::HIS4*, *PNSI-3::*(P_{GAP}-*CAS9*-T_{DAS1}), *PNSI-4::*(P_{GAP}-*RAD52*-T_{AOX1}), *PNSIII-6::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSIII-8::*(P_{AOX1}-*BaAspDH*-T_{CAT1}), *PNSIV-7::* (P_{AOX1}-*BsADC*-T_{KpMDH3}), *PNSII-8::*(P_{AOX1}-*BsADC*-T_{KpMDH3}), *ald4Δ*, *PNSIII-12::*(P_{AOX1}-*BcBAPAT*-T_{FBP1}), *PNSIV-11::*(P_{S2}-*EcYDFG*-T_{FBP1})	This study

3. Conclusion

In summary, we identified and modified a promoter, P_{S2}, which exhibits moderate expression strength in methanol medium, and the strong intensity up to 90 % of P_{GAP}. Furthermore, we constructed a hybrid biosynthetic pathway for 3-HP by integrating the β-alanine pathway and the MCR pathway using the promoters P_{AOX1} and P_{S2}. The maximum production of 3-HP reached to 23 g/L. Therefore, *P. pastoris* serves as a favorable cell factory platform for the methanol bioconversion of chemicals.

4. Methods and materials

4.1. Strain, plasmids and medium

The strains, plasmids and primers used in the article were listed in Table S3, Table S4 and Table S5, respectively. *P. pastoris* strain was cultured in YPD medium containing 20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose. G418 antibiotic (200 mg/mL) was added to screen the transformants. YPM medium containing 20 g/L peptone, 10 g/L yeast extract, 20 g/L methanol. The *Escherichia coli* was grown in Luria Bertani (LB) containing 10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, and added 50 mg/mL kanamycin to screen the transformants. Minimal medium (Delft) containing 2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 2 mL/L trace metals, and 1 mL/L vitamin solution [57] was adopted to cultivate *P. pastoris* strains. The promoters were screened with 20 g/L glucose and 10 g/L methanol with 100 mg/L histidine if necessary, respectively. For all Delft medium, the initial pH was adjusted to 5.6 by KOH. Solidified plates containing 2 % agar were used for colony selection. All yeast strains were cultivated at 30 °C, 220 rpm except for special conditions, and all *E. coli* strains were cultured at 37 °C, 220 rpm in a shake incubator (Zhichu Shaker ZQZY-CS8).

The batch medium [50] was used as the initial medium for fermentation in bioreactor, which contain 40 g/L glycerol, 0.02 g/L $CaCl_2{\cdot}2H_2O$, 1.8 g/L citric acid, 0.9 g/L KCl, 12.6 g/L $(NH_4)_2HPO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$ and 50 μL antifoam 204 (Sigma), 0.4 mg/L biotin, 2 ml/L vitamin solution, 5 ml/L trace metal. The pH level was set to 5.6 using 5 M HCl. After being filtered and sterilized, the vitamin solution, biotin and trace metal mixture were incorporated into the sterilized medium once cooled down. The feeding salts containing 0.35 g/L $CaCl_2{\cdot}2H_2O$, 6.45 g/L $MgSO_4{\cdot}7H_2O$, 10 g/L KCl, 200 μL antifoam 204, 1.2 mg/L biotin, 6 mL/L vitamin solution, 5 mL/L trace metal and was prepared at 2× concentration for feeding in bioreactor fermentation. $CaCl_2{\cdot}2H_2O$ was sterilized, vitamin solution and biotin trace metal were filtered, and added to autoclave feeding media after cooling.

4.2. Screening candidate promoters

EMP, TAC and PPP pathway related genes of *P. pastoris* were collected from KEGG and NCBI database, and 1000 bp upstream of the start codon ATG were captured as potential promoters [58,59]. Samples were taken at the logarithmic stages (16 h, 20 h, 24 h) and the stationary stages (48 h, 72 h).

4.3. Genetic manipulation

Most of genes manipulation were accomplished by the use of our previously reported CRISPR/Cas9 gene editing system in *P. pastoris* [6]. Gene expression cassettes, containing 1000 bp upstream and downstream sequences separately, were fused by overlapping extended PCR [60]. Then exchanged with neutral sites on the genome by homologous recombination with 80 μL competent cell, 500 ng donor and 300 ng gRNA plasmid through electrotransformation [6,61]. All endogenous gene fragments were amplified from PC111 genome DNA and the chassis strains were the strain PC111 [6] and G4RCH. All exogenetics gene sequences were listed in Table S6.

4.4. Construction of hybrid promoters

The UAS region of promoter was determined by using the predicted core promoter region (BDGP: Neural Network Promoter Prediction (fruitfly.org)), and then reasonably divided into four fragments according to the predicted transcription factor binding sites (Alibaba2 (gene-regulation.com)) by a stepwise truncation strategy [29].

Hybrid promoter Y1-eGFP expression cassettes was constructed by double digestion homologous recombination using clonExpress MultiS One Step Cloning Kit (Vazyme, China), and the expression cassette was amplified from the plasmid pUC19-Y1. The restriction enzyme sites *Bam*HI/*Hin*dIII were used to digest the plasmid and insert fragments. Other eGFP expression cassettes were constructed by homologous recombination overlapping extension PCR based on CRISPR/Cas9 system, and all promoters were integrated into the *PNSII-5* site of *P. pastoris* to characterize the promoter strength.

4.5. Fluorescence signal assay

Positive colonies were firstly streaked on YPD plates and grown for 3 days, then precultured into 3 mL YPD medium and transferred into Delft-histidine medium contained 20 g/L glucose (OD_{600} = 0.1) or 10 g/L methanol (OD_{600} = 0.2) as carbon source, separately. Samples were

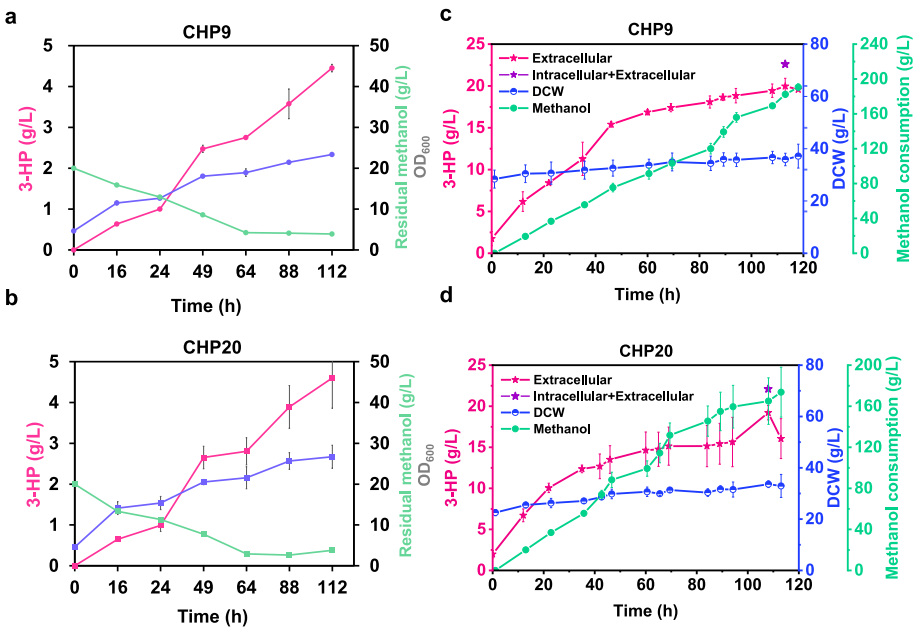

Fig. 4. Fed-batch fermentation of the final engineered strains CHP9 and CHP20 for 3-HP production. Fed-batch fermentation of the engineered strains CHP9 **(a)** and CHP20 **(b)** in shake flasks. Fed-batch fermentation of the strains CHP9 **(c)** and CHP20 **(d)** in 1 L bioreactors. Time courses of intracellular and extracellular 3-HP titers (in purple five-pointed star), extracellular 3-HP titers (in magenta), concentration of dry cell weight or growth curves (in blue), and concentration of methanol or methanol consumption (in green) are shown.

harvested at certain time interval, and the supernatant were removed by centrifugating at 6000 rpm for 5 min. Then the OD_{600} was diluted to 1 with water to normalized the OD_{600} value, and then 200 μL cell suspension was added to a 96-well plate, and fluorescence Rfu/OD_{600} was detected by Tecan SPARK enzyme marker (Tecan, Switzerland). The excitation and emission wavelengths were 485 and 525 nm [6].

4.6. Evaluation of 3-HP production in engineered strain

Fermentation for production of β-alanine and 3-HP in 100 mL shake flask with 20 g/L methanol in Delft medium, and the strains were cultured at 30 °C, 220 rpm. 1 mL samples were taken to measure the concentration of β-alanine or 3-HP at 96 h or 120 h. The fed batch fermentation for 3-HP production was performed in 250 mL shake flask containing 50 mL Delft medium, the primary seed was cultured in YPD medium and the secondary seed was fed in YPM medium to induce the enzyme expression of the methanol pathway in advance, and the initial OD_{600} was adjusted to 5 for fermentation. Besides, 1 mL 10×Delft was feeded and pH was set to 5, and methanol was supplemented into culture to maintain the concentration at 5 g.

The fed batch fermentation was performed in 1 L bioreactor with 500 mL working volume (T&J-Minibox6 1.5L*8 Intelli-Ferm). The cultivation was divided into two stages, with first stage, the initial batch stage was inoculated at an initial OD_{600} of 1 in batch medium, and entered into next stage after DCW was reached to about 35 g/L. Second stage, the feeding salts medium fed rate was maintained at 0.3–0.5 mL/h. The concentration of methanol maintained at 5 g/L in culture. All parameters containing the pH, temperature, dissolved oxygen (DO) level, aeration were real-time monitoring using parallel bioreactors. The lower limit pH was kept at 5 by 15 % ammonia, temperature was controlled at 28 °C, and the aeration set to 0.5 and increased to 1 with agitation at 400 rpm–800 rpm gradually increasing depending on the DO level throughout the fermentation process [46,50]. Samples were taken every 5 h or 12 h to measured OD_{600}, methanol and production in time.

1 mL Samples were centrifuged at 1300 rpm for 5 min, and the supernatant was filtered with 0.2 μm water membrane to determine the extracellular concentration of β-alanine and 3-HP. And the cell pellet was resuspended in 300 μL of ddH_2O with moderate 0.5 mm beads and, followed by thorough grinding to disrupt the cells. An additional 700 μL of ddH_2O was added and mixed. The mixture was then centrifuged, and the supernatant was collected for the determination of intracellular 3-HP concentration. The pre-column derivation method was used for determination of β-alanine. The β-alanine were derived using derivation agent *o*-phthalaldehyde (OPA) via the online derivative method [62], and the concentration was measured by high-performance liquid chromatography (HPLC) carrying Agilent ZRABOX SB-C18 column (4.6 mm × 250 mm, 5 μm) with UV detector at 338 nm. The mobile phase contains 700 mL 35 mM CH_3COONa (pH = 7) and 300 mL 30 % methanol at a flow rate of 1 mL/min at 40 °C for 20 min [47,63]. The concentration of 3-HP was determined using an Aminex HPX87G column from Bio-Rad with 210 nm by Agilent 1260 infinity II HPLC (Agilent, America), the mobile phase was 5 mM H_2SO_4 at a flow rate of 0.5 mL/min, and temperature of 50 °C for a duration of 30 min, the UV detector was used for 3-HP measuring, and RID for methanol measuring.

4.7. Statistical analysis

All statistical analysis was carried out by Microsoft Excel software. All data were presented as the means ± SD. Experiment was performed in three biological parallels.

CRediT authorship contribution statement

Shushu Chen: Writing – original draft, Methodology, Investigation, Formal analysis. **Mengyao Zhang:** Methodology, Investigation. **Xiaoyan Wu:** Methodology, Investigation, Formal analysis, Conceptualization. **Fan Bai:** Writing – review & editing, Methodology, Investigation. **Linhui Gao:** Methodology, Investigation, Formal analysis. **Yiwei Shen:** Methodology, Formal analysis, Data curation. **Shaohua Dou:** Supervision, Investigation, Conceptualization. **Peng Cai:** Writing – review & editing, Project administration, Methodology, Funding acquisition. **Yongjin J. Zhou:** Writing – review & editing, Supervision, Funding acquisition.

Availability of data and material

Data that supports the finding of this study are freely available in the main text and the supplementary materials.

Declaration of competing interest

Yongjin J. Zhou is an Associate Editor for *Synthetic and Systems Biotechnology* and was not involved in the editorial review or the decision to publish this article. All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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