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Overproduction of 3-hydroxypropionate in a super yeast chassis

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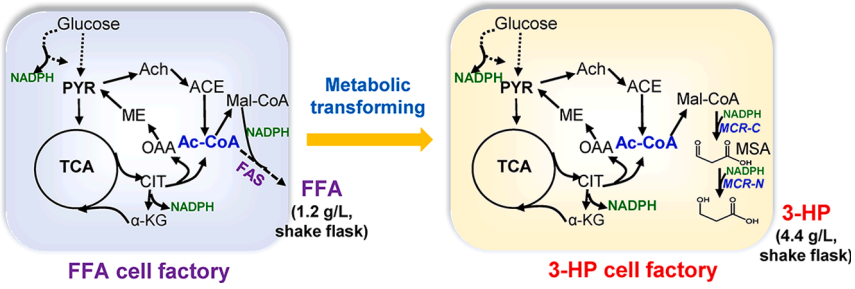
HIGHLIGHTS

- 3-HP production was significantly improved in a super yeast chassis;
- Optimizing the expression of Mcr was beneficial for 3-HP production;
- Stable expression of *MCR-C* enabled the highest 3-HP production of 56.5 g/L.

GRAPHICAL ABSTRACT



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ABSTRACT

3-Hydroxypropionate (3-HP) is a platform chemical for production of acrylic acid, acrylamide and biodegradable polymers. Several microbial cell factories have been constructed for production of 3-HP from malonyl-CoA by using a malonyl-CoA reductase, which however suffer from inadequate supply of precursor and cofactor. Here 3-HP biosynthesis was optimized in a super yeast chassis with sufficient supply of precursor malonyl-CoA and cofactor NADPH, which had a 3-fold higher 3-HP (1.4 g/L) than that of wild-type background. The instability of the engineered strain was observed in fed-batch fermentation due to the plasmid loss, which may be caused by the toxic intermediate malonate semialdehyde. Genome integration of *MCR-C* encoding C-terminal of *MCR* enabled stable gene expression and much higher 3-HP production of 4.4 g/L under batch fermentation and 56.5 g/L under fed-batch fermentation with a yield of 0.31 g/g glucose. This was the highest 3-HP production reported from glucose in engineered microbes.

1. Introduction

3-Hydroxypropionate (3-HP) is a platform chemical that can be used for producing a series of value-added chemicals, such as acrylic acid, acrylamide and biodegradable polymers (Choi et al., 2015; Nguyen-Vo et al., 2022; Son et al., 2022). 3-HP can be biosynthesized from various precursors such as malonyl-CoA (Liu et al., 2016), glycerol (Kumar & Park, 2018), 1,3-propanediol (Jiang et al., 2021), β-alanine (Borodina et al., 2015) or oxaloacetate (Tong et al., 2021), among which malonyl-CoA is considered as an ideal precursor for 3-HP production since its regulation and engineering strategies are comprehensively studied (Liu et al., 2017).

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Biosynthesis of 3-HP from malonyl-CoA is catalyzed by a bifunctional enzyme malonyl-CoA reductase (Mcr). Previous studies showed that dissecting Mcr from *Chloroflexus aurantiacus* into Mcr-C (C terminal of Mcr catalyzing malonyl-CoA to malonate semialdehyde, amino acids 550-1219) and Mcr-N (N terminal of Mcr catalyzing malonate semialdehyde to 3-HP, amino acids 1-549), improved 3-HP production in *Escherichia coli* (Liu et al., 2013). Further mutation of Mcr-C (N940V, K1106W and S1114R) increased the overall catalytic activity (Liu et al., 2016). Thus, engineering Mcr should be helpful for improving 3-HP production in engineered microbes.

Malonyl-CoA based 3-HP biosynthesis requires sufficient supply of precursor malonyl-CoA and cofactor NADPH, which can be realized by engineering the central metabolism (Zhou et al., 2016). However, global metabolic rewiring always involves time-consuming and labor-intensive multiple rounds of genetic manipulation. Fortunately, engineering *Saccharomyces cerevisiae* for high-level production of free fatty acids (FFA) by extensively rewiring the cellular metabolism has been done in previous work (Yu et al., 2018). Since the biosynthesis of FFA and 3-HP shared the same precursor malonyl-CoA and cofactor NADPH, here metabolically transforming this super yeast for high-level 3-HP production by optimizing the 3-HP biosynthetic pathway was performed. This metabolically transformed chassis should be beneficial for 3-HP production with enhanced supply of malonyl-CoA and NADPH. It was also observed that the plasmid expression of *MCR-C* suffered from serious strain instability due to plasmid loss, and genomic integration guaranteed the stability of gene expression and resulted in 56.5 g/L of 3-HP production, the highest production from glucose in microbial cell factories.

2. Materials and methods

2.1. Strains, media, and reagents

E. coli strain DH5α was used for plasmid construction and amplification. *S. cerevisiae* strains and plasmids were listed (see supplementary materials). The flowchart of yeast strain construction was described (see supplementary materials). *S. cerevisiae* strains were cultured at 30°C in YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) or SD medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose supplemented with 20 mg/L histidine and/or 20 mg/L uracil if needed). 20 g/L agar was added for preparing solid media.

2.2. Genetic manipulation

A *CAS9* expression cassette fused with *KanMX* (P_{TEF1}-*CAS9*-T_{CYC1}-*KanMX*) was amplified from plasmid pECAS9-kanMX-gRNA and

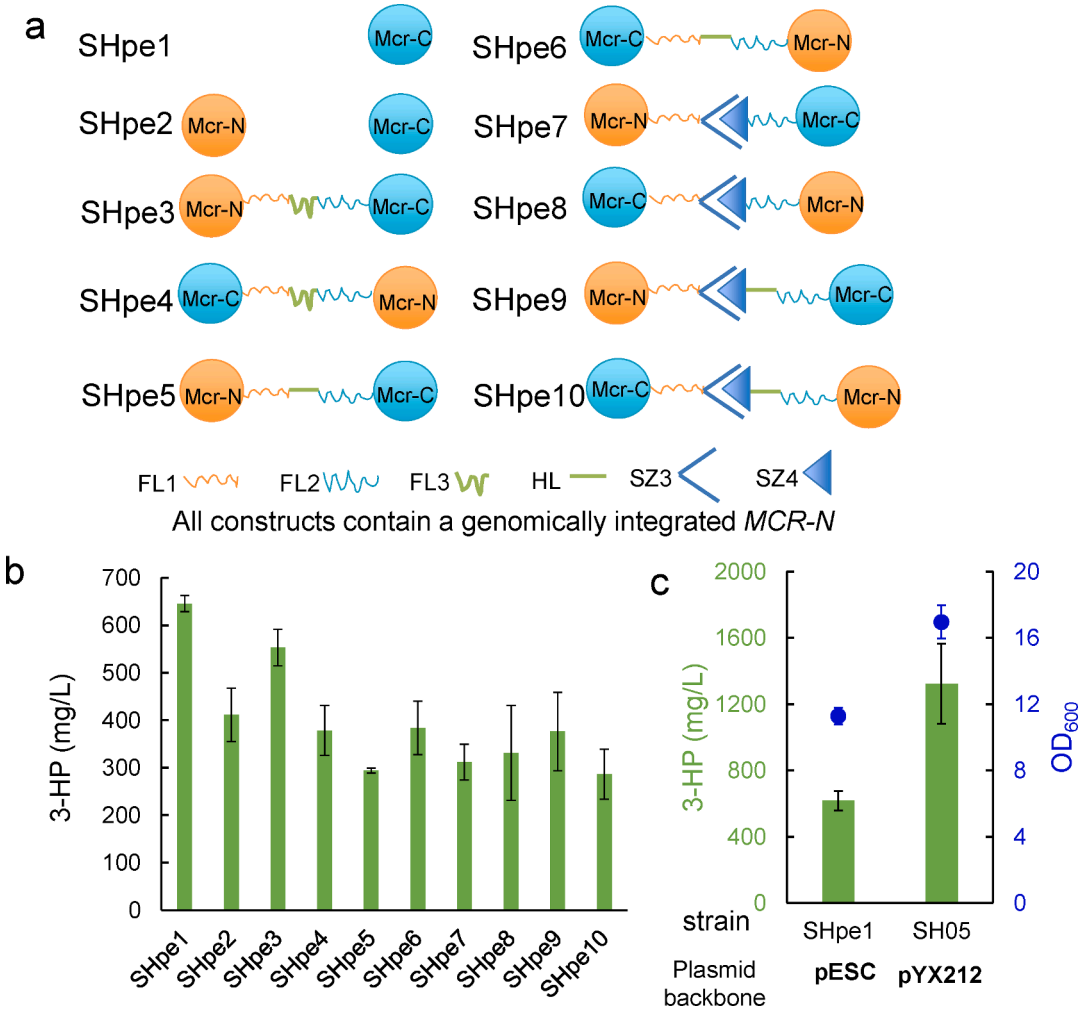

Fig. 1. Optimization of Mcr expression to promote 3-HP synthesis. (a) Different enzyme expression patterns: SHpe1 and 2, separated Mcr-C and Mcr-N; SHpe3-6, enzyme fusion; SHpe7-10, enzyme affinity. FL, flexible linker, FL1: GPGRPPPGRG; FL2: ASGAGGSEGGGSEGGTSGAT; FL3: TDGASSS. HL, rigid linker, GGAGAAGAAG. SZ, synthetic zipper, SZ3: NEVTTLENDAAFIENENAYLEKEIARLRKEKAALRNRLAHKK; SZ4: MQKVAELKNRVAVKLNRNEQLKNKVEELKNRNAYLKNELATLENEVARLENDVAE. (b) 3-HP production in the engineered strains with different expression patterns of Mcr. (c) 3-HP production and cell growth with different plasmids that were used for MCR-C expression.

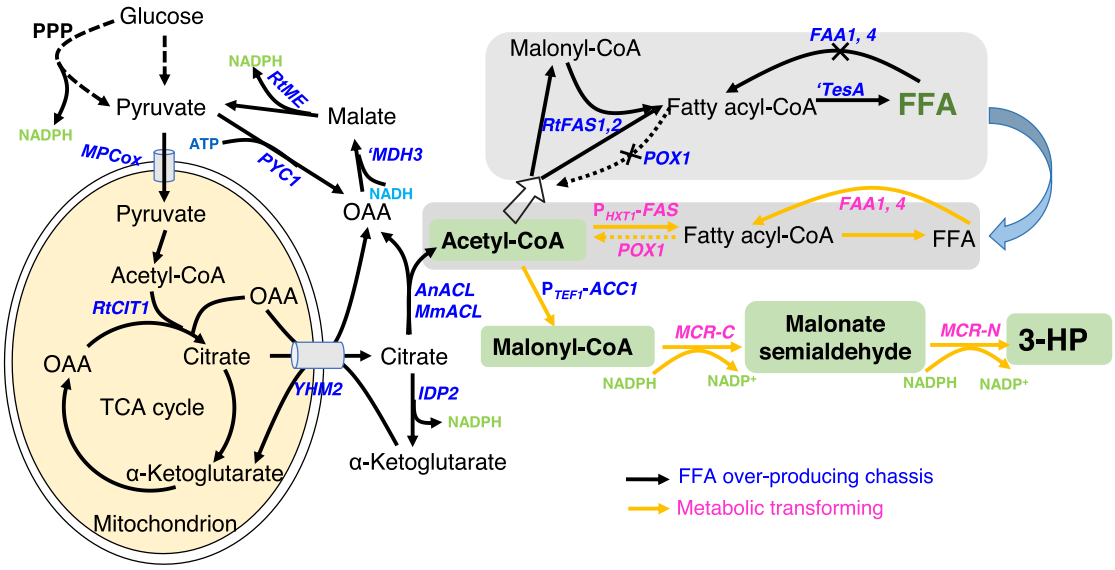

Fig. 2. Metabolic transforming from FFA production to 3-HP biosynthesis. The background strain Y&Z036 was an FFA overproducing strain with enhanced supply of precursor acetyl-CoA and malonyl-CoA, and cofactor NADPH, which were also required for 3-HP synthesis. The overexpressed *RtFAS1/4* and *'TesA* genes in Y&Z036 were deleted, and the deleted *FAA1/4* and *POX1* genes were *in situ* recovered, preventing FFA accumulation. *MCR* gene was introduced for 3-HP synthesis. *MPCox*: mitochondrial pyruvate importer gene; *RtCIT1*: citrate synthase gene from *Rhodosporidium toruloides*; *YHM2*: mitochondrial citrate exporter gene; *AnACL*: ATP:citrate lyase gene from *Aspergillus nidulans*; *MmACL*: ATP:citrate lyase gene from *Mus musculus*; *IDP2*: $NADP^+$-dependent isocitrate dehydrogenase gene; *RtME*: malic enzyme gene from *R. toruloides*; *'MDH3*: malate dehydrogenase gene with removed peroxisomal signal; *PYC1*: pyruvate carboxylase gene 1; *RtFAS1/2*: fatty acid synthase gene 1 and 2 from *R. toruloides*; *FAA1/4*: long-chain fatty acid-CoA ligase gene 1 and 4; *POX1*: acyl-CoA oxidase gene 1; *'TesA*: acyl-CoA thioesterase gene; *ACC1*: acetyl-CoA carboxylase gene; *MCR-C*: C terminal of malonyl-CoA reductase gene; *MCR-N*: N terminal of malonyl-CoA reductase gene.

integrated at the *XI-5* site (Mikkelsen et al., 2012) in wild-type strain CEN. PK 113-11C. The strain with *KanMX* knock-out was named as XC01. All guide-RNAs (gRNAs) were designed by the CHOPCHOP webtool (http://chopchop.cbu.uib.no), and gRNA-expressing plasmids were constructed according to described methods (Mans et al., 2015; Yang et al., 2020). The donor DNAs for gene deletion and integration were assembled by one-pot fusion PCR. All primers used in this study were listed (see supplementary materials). The *MCR* gene (N940V, K1106W and S1114R) from *C. aurantiacus* was codon optimized for *S. cerevisiae* (see supplementary materials) and synthesized by Exsyn-bio Technology Co., Ltd, Shanghai, China. The *MCR* gene was dissected into *MCR-N* (1-549) and *MCR-C* (550-1219). To optimize Mcr enzyme expression, three flexible linkers (FL1, FL2, FL3) and a rigid linker (HL) were used for protein fusion, or synthetic zippers (SZ3 and SZ4) (Reinke et al., 2010) were used to increase protein affinity for substrate channeling.

2.3. Strain construction

Gene expression cassettes for genome integration were illustrated (see supplementary materials). For 3-HP production, central metabolism engineering was conducted in strain Y&Z036 (Yu et al., 2018), and the modifications were as follows: *HIS3*, *'TesA* and *RtFAS1*, *RtFAS2* genes were deleted; the *FAA1/4* and *POX1* genes were *in situ* restored. The 3-HP biosynthetic pathway optimization was conducted as follows: *MCR-N* under the control of the promoter P_{GAL7} was integrated into *XI-1* site; *MCR-C* and *MCR-N* under the control of the promoter P_{TDH3}, respectively, were integrated into *XII-3* site; two extra copies of *MCR-C* under the control of the bi-directional promoter $P_{GAL1,10}$ were integrated into *XII-5* site. To replace *FAS1* native promoter with *HXT1* promoter, 300 bp upstream of *FAS1* open reading frame (ORF) was replaced with P_{HXT1}. For *in situ* recovering selection markers *HIS3* and *URA3*, functional ORF and its upstream 500 bp and downstream 500 bp were fused and integrated into native site by using homologous recombination.

2.4. Batch and fed-batch fermentation

The batch fermentation was conducted in 100 mL shake flasks containing 20 mL minimal medium: 2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \bullet 7H_2O$, 20 g/L glucose, 2 mL/L trace metal and 1 mL/L vitamin solutions (Verduyn et al., 1992). The yeast cells were cultivated at 30°C, 220 rpm for 4 days with an initial inoculation OD_{600} of 0.2. As *GAL80* gene was deleted in the engineered yeast strains, no galactose was needed for *GAL* promoter activation, enabling repression of *MCR* genes under the control of *GAL* promoter (P_{GAL1}, P_{GAL7} and P_{GAL10}) at high glucose concentration and high expression at low glucose level.

The fed-batch fermentation in bioreactors was conducted with an initial working volume of 0.4 L minimal medium using a DasGip Parallel Bioreactors System. The initial inoculation OD_{600} was 0.4. The temperature was set as 30°C, and initial agitation speed was set as 800 rpm and increased to maximal 1,200 rpm depending on the dissolved oxygen (DO) level. Aeration was initially set as 36 sL/h and increased to maximal 48 sL/h depending on the DO level. The DO level was maintained above 30%, and the pH was kept 5.6 by automatic addition of 4 M KOH or 2 M HCl. During the fed-batch cultivation, the cells were fed with a medium (500 g/L glucose solution, 10 g/L $(NH_4)_2SO_4$, 57.6 g/L KH_2PO_4, 2 g/L $MgSO_4 \bullet 7H_2O$, 10 mL trace metal and 5 mL vitamin solutions) in a pulse feeding strategy. The pulse feed (T=4 h before 46 h and T= 6 h after 46 h) were used and the feeding rate was kept fast at a short time (feeding 15 min with 50 mL/h every period) to avoid glucose and ethanol accumulation. After fermentation, the samples were centrifuged at 13000×*g* for 5 min and the supernatants were subject to quantification of glucose, ethanol and 3-HP.

2.5. 3-HP quantification

The extracellular 3-HP were determined by high-performance liquid chromatography (Shimadzu LC-2030, Japan) system equipped with an Aminex HPX-87H column (Bio-Rad) and RID, VWD detectors. UV detection was performed at 210 nm. The samples were eluted with 5 mM H_2SO_4 at a flow rate of 0.5 mL/min at 65°C for 30 min. The

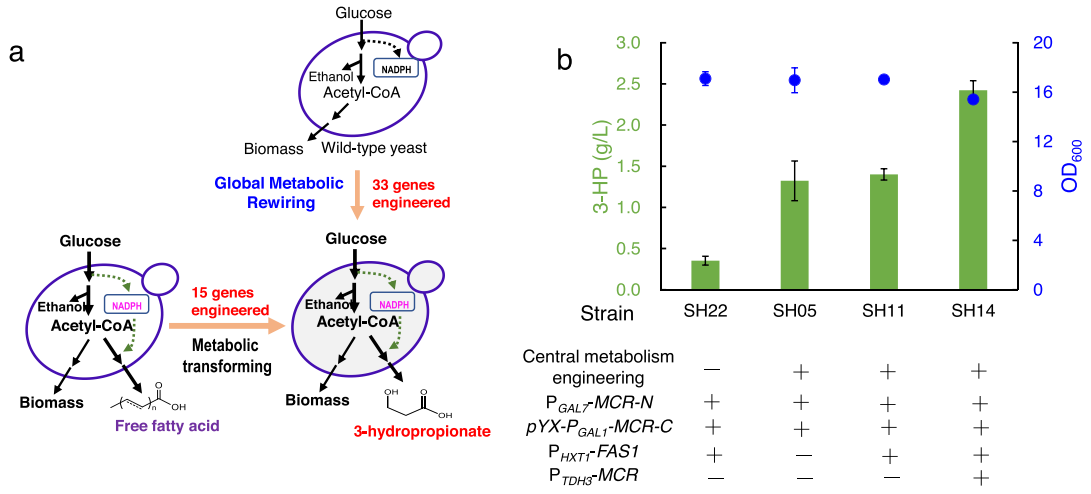

Fig. 3. Engineering central metabolism promoted 3-HP production. (a) Metabolic transforming of an FFA-overproducing yeast, with enhanced supply of precursor acetyl-CoA and cofactor NADPH, avoided the manipulation of 18 genes and provided a super chassis for 3-HP production. FFA, free fatty acids. (b) 3-HP production from wild-type background and super chassis. Mean value and standard deviation were shown (n = 3).

concentration of the extracellular glucose and ethanol were determined using biosensor analyzer (SBA-40D, Shandong, China) to monitor fermentation process. The results were expressed as mean ± standard deviation (SD) of three replicates.

3. Results and discussion

3.1. Optimization of MCR expression for 3-HP production

Since it has been reported that dissecting Mcr into two enzymes improved 3-HP production and the Mcr-C (catalyzing malonyl-CoA to malonate semialdehyde) is a rate-limiting enzyme (Liu et al., 2016), expressing *MCR-C* in a high-copy plasmid pESC was performed here, while *MCR-N* was integrated into genome (Fig. 1a). The resulting strain SHpe1 produced 645 mg/L 3-HP (Fig. 1b). Then several Mcr variants were constructed by fusing or separated expression of *MCR-C* and *MCR-N* in plasmid pESC. The affinity peptides and flexible linkers were also used for enzyme fusion to avoid the enzyme inference (Fig. 1a). Unfortunately, all constructs had lower 3-HP production compared to SHpe1 (Fig. 1b), which suggested that a separated expression of Mcr-C and Mcr-N with higher expression level of Mcr-C was beneficial for 3-HP production in *S. cerevisiae*. In addition, another plasmid pYX212 (Zhou et al., 2016) was also used for expression of Mcr-C, which resulted in 2.2-fold higher 3-HP (1.3 g/L) than that of pESC (Fig. 1c). The cell growth of the strain SHpe1 containing pESC was 33% lower than that of the strain SH05 containing pYX212 (Figure 1c), which may be the reason for low 3-HP production of SHpe1. The selection markers in these two plasmids were different: pESC contained *HIS3* while pYX212 contained *URA3*, which might cause the difference of cell growth and 3-HP production.

3.2. Engineering central metabolism improved 3-HP production

3-HP biosynthesis requires equal amount of malonyl-CoA and double amounts of NADPH, thus the supply of malonyl-CoA and NADPH might be a bottleneck for high-level production of 3-HP. Extensively rewiring the central metabolism should be helpful for enhancing the supply of malonyl-CoA and NADPH as was done previously for overproduction of FFA (Yu et al., 2018; Zhou et al., 2016). In detail, *MPCox* (mitochondrial pyruvate carrier gene), *RtCIT1* (citrate synthase gene from *Rhodosporidium toruloides*), *YHM2* (mitochondrial citrate-2-oxoglutarate transporter gene), *MmACL/AnACL* (ATP: citrate lyase gene from *Mus musculus* and *Aspergillus nidulans*) were overexpressed to enhance cytosolic acetyl-CoA supply; *ACC1* (acetyl-CoA carboxylase gene) was overexpressed to enhance malonyl-CoA supply; and *'MDH3* (malate dehydrogenase gene with removed peroxisomal signal), *RtME* (malic enzyme gene from *R. toruloides*), *PYC1* (pyruvate carboxylase gene), *IDP2* ($NADP^+$-dependent isocitrate dehydrogenase gene), *ZWF1* (glucose-6-phosphate dehydrogenase gene), *GND1* (phosphogluconate dehydrogenase gene), *TKL1* (transketolase gene), *TAL1* (transaldolase gene) were overexpressed to enhance NADPH supply (Fig. 2). These strategies significantly improved the production of malonyl-CoA derived product FFA (Yu et al., 2018), which however were time consuming and laborious due to manipulation of 33 genes from wild-type background. Alternatively, metabolically transforming the super FFA strain toward 3-HP production was conducted here (Fig. 2 and supplementary material). *In situ* recovery of the deleted *FAA1/4* and *POX1*, and removing the overexpressed genes (*RtFAS1/2 and 'TesA*) for FFA biosynthesis created a chassis strain SH03 with efficient supply of malonyl-CoA and NADPH (Fig. 2 and 3a). Introduction of *MCR* with *MCR-C* under the control of the promoter P_{GAL1} in plasmid pYX212 and *MCR-N* under the control of the promoter P_{GAL7} in genome into this super chassis enabled 3-HP production of 1.3 g/L (strain SH05, Figure 3b). To relieve the competition of malonyl-CoA and NADPH, the fatty acid synthase gene *FAS1* was expressed under the control of a glucose concentration sensitive promoter P_{HXT1} (see supplementary materials), enabling down-regulation of *FAS1* under glucose-limited conditions (David et al., 2016). Although *FAS1* engineering only marginally improved 3-HP titer (SH11, 1.4 g/L) in shake flask batch fermentation (Figure 3b), this strategy may be more helpful in the long-term fed-batch fermentation considering its effect of uncoupling cell growth and product synthesis. In comparison, the central metabolism engineering strain SH11 produced 3-fold higher 3-HP than that of the wild type background strain expressing the same *MCR* (SH22, 0.35 g/L). This result demonstrated that enhancing the supply of malonyl-CoA and NADPH released the potential of 3-HP biosynthesis in *S. cerevisiae*. Genome integration of another copy of *MCR* under control of the promoter P_{TDH3} (P_{TDH3}-*MCR-C* and P_{TDH3}-*MCR-N*) further improved 3-HP to 2.4 g/L (SH14), a 71% improvement compared with strain SH11 (Fig. 3b). It was supposed that promoter P_{GAL} and P_{TDH3} enabled *MCR* expression throughout the fermentation process for enhancing the 3-HP production.

3.3. Plasmid loss in fed-batch fermentation

Fed-batch fermentation was conducted to evaluate the performance of strain SH14h, a prototrophic strain of SH14 with *in situ* recovery of

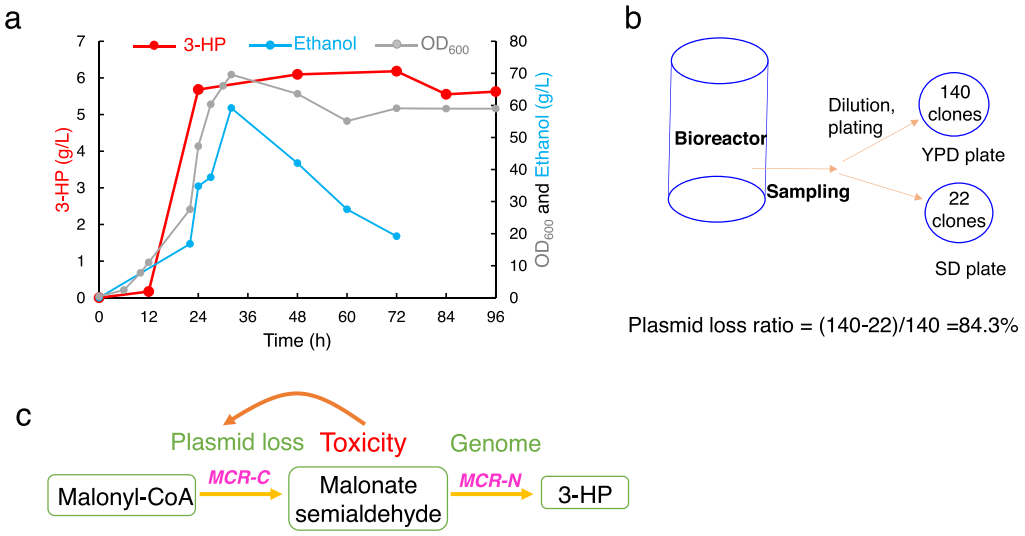

Fig. 4. Plasmid loss in fed-batch fermentation of SH14h in bioreactors. (a) Fed-batch fermentation of SH14h in 1 L bioreactor with an exponential feeding strategy. (b) The fermentation broth was sampled from the bioreactor and plated after dilution on YPD and SD plates. The plasmid losing clones did not grow in selective SD media, but could grow on YPD plates. In this case, the plasmid loss rate can be calculated as (140-22)/140 = 84.3%. (c) The reaction from malonyl-CoA to 3-HP catalyzed by Mcr-C and Mcr-N. The intermediate malonate semialdehyde was toxic to yeast, which forced the loss of the plasmid containing *MCR-C*.

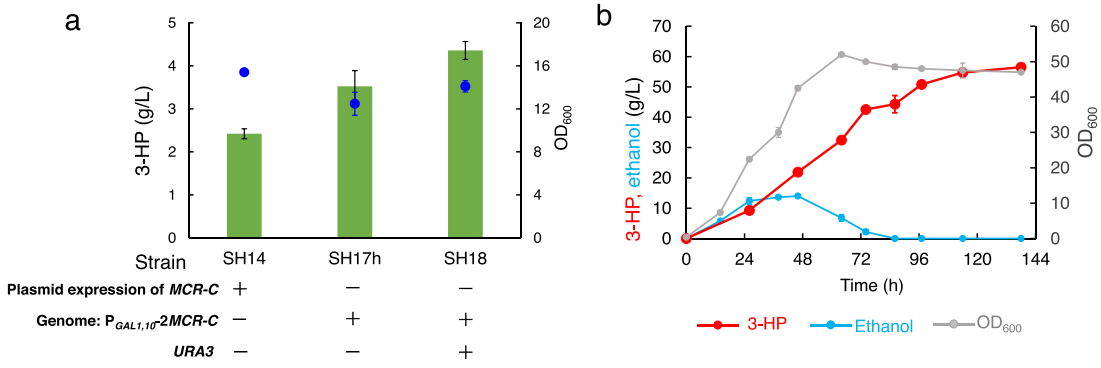

Fig. 5. Stable *MCR-C* expression in genome for improving 3-HP production. (a) Genome integration of *MCR-C* and *in situ* recovery of *URA3* promoted 3-HP production. (b) Fed-batch fermentation of stable strain SH18 in 1-L bioreactor with a pulse-feeding strategy. 3-HP production, ethanol accumulation and biomass concentration were determined to monitor the fermentation process.

selection marker gene *HIS3*. This strain almost reached the highest 3-HP production within 24 h and stopped to accumulate 3-HP after 24 h, regardless of continuous consumption of glucose and ethanol (Fig. 4a). It was observed that 84.3% of the total cells lost the *MCR-C* expressing plasmid (Fig. 4b). It was speculated that the toxic intermediate malonate semialdehyde which was catalyzed by Mcr-C forced plasmid loss in the fermentation (Fig. 4c). Therefore, much attention should be paid to maintaining the plasmid stability in *S. cerevisiae* in the large-scale fermentation.

3.4. Construction of a stable strain for high level production of 3-HP

For construction of a stable strain, optimizing the expression of *MCR-C* through genome integration was performed. Considering the requirement of high expression level of *MCR-C*, a bi-directional promoter $P_{GAL1,10}$ was used to express *MCR-C*, enabling simultaneous integration of two copies of *MCR-C* at the genome neutral site XII-5 (Mikkelsen et al., 2012). The resulting strain SH17h produced 3.5 g/L 3-HP in shake flasks, 45% higher than that of the strain SH14 with plasmid expressing *MCR-C* (Fig. 5a). Further, *in situ* recovery of selection marker *URA3* in SH17h generated strain SH18, which produced 4.4 g/L 3-HP in shake flask (Fig. 5a). Fed-batch fermentation of SH18 had continuous 3-HP accumulation throughout the whole fermentation process even though the strain stopped to grow at 64 h, which suggested that the *MCR* was stably expressed for 3-HP biosynthesis. The final 3-HP production reached 56.5 g/L with a yield of 0.31 g/g glucose (41.3% of maximal theoretical yield) and a productivity of 0.53 g/L/h (Fig. 5b), which was the highest 3-HP production from glucose to our knowledge (see supplementary materials) (Borodina et al., 2015; Hellgren et al., 2020; Kildegaard et al., 2016; Liu et al., 2016; Qin et al., 2020; Takayama et al., 2018; Tong et al., 2021). The result demonstrated that stable *MCR* expression in the super yeast chassis via metabolic transforming released the great potential of 3-HP production.

4. Conclusion

Optimization of an Mcr pathway in a super yeast chassis with enhanced supply of precursor and cofactor, enabled the highest production of 3-HP (56.5 g/L). Metabolic transforming an FFA overproducing strain toward 3-HP biosynthesis significantly improved 3-HP production with saving multiple rounds of genetic manipulation. Plasmid-based expression of the *MCR-C* resulted in the plasmid loss and

low 3-HP production, and genomic integration guaranteed stable gene expression and catalytic activity in long-run fed-batch fermentation. This study demonstrated that the central metabolism and biosynthetic pathway should be carefully optimized and adapted for stable and high-level production of chemicals.

CRediT authorship contribution statement

Wei Yu: Methodology, Validation, Investigation, Formal analysis, Writing – original draft. **Xuan Cao:** Methodology, Investigation. **Jiaoqi Gao:** Methodology, Writing – original draft. **Yongjin J. Zhou:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors have filed a patent (202111531540.9) for protecting the production of 3-hydroxypropionate in yeast.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127690.

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