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Original Research Article

A novel CRISPR/Cas9 system with high genomic editing efficiency and recyclable auxotrophic selective marker for multiple-step metabolic rewriting in *Pichia pastoris*

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ABSTRACT

The methylotrophic budding yeast *Pichia pastoris* has been utilized to the production of a variety of heterologous recombinant proteins owing to the strong inducible alcohol oxidase promoter (pAOX1). However, it is difficult to use *P. pastoris* as the chassis cell factory for high-valuable metabolite biosynthesis due to the low homologous recombination (HR) efficiency and the limitation of handy selective markers, especially in the condition of multistep biosynthetic pathways. Hence, we developed a novel CRISPR/Cas9 system with highly editing efficiencies and recyclable auxotrophic selective marker (HiEE-ReSM) to facilitate cell factory in *P. pastoris*. Firstly, we improved the HR rates of *P. pastoris* through knocking out the non-homologous-end-joining gene (*Δku70*) and overexpressing HR-related proteins (*RAD52* and *RAD59*), resulting in higher positive rate compared to the basal strain, achieved 97%. Then, we used the uracil biosynthetic genes *PpURA3* as the reverse screening marker, which can improve the recycling efficiency of marker. Meanwhile, the HR rate is still 100% in uracil auxotrophic yeast. Specially, we improved the growth rate of uracil auxotrophic yeast strains by overexpressing the uracil transporter (*scFUR4*) to increase the uptake of exogenous uracil from medium. Meanwhile, we explored the optimal concentration of uracil (90 mg/L) for strain growth. In the end, the HiEE-ReSM system has been applied for the inositol production (250 mg/L) derived from methanol in *P. pastoris*. The systems will contribute to *P. pastoris* as an attractive cell factory for the complex compound biosynthesis through multistep metabolic pathway engineering and will be a useful tool to improve one carbon (C1) bio-utilization.

1. Introduction

The non-conventional yeast *P. pastoris* (syn. *Komagataella phaffii*), has been widely used as the platform for heterologous protein expression and biopharmaceuticals because of its rapid growth rate, minimized protein glycosylation and high cell density cultivation [1]. Nowadays, methanol has attracted a lot of attention for biotransformation as a nonfood one carbon (C1) feedstock [2], which can be obtained from sources such as natural gas or CO_2 [3]. *P. pastoris*, a representative methylotrophic yeast, is capable to assimilate methanol as the sole carbon source for growth, as well as capable to oxidize methanol to generate energy. Meanwhile, as a canonical Crabtree-negative yeast, *P. pastoris* exhibits a higher carbon/metabolic flux of pentose phosphate pathway (PPP), which can provide more NADPH generation per carbon compared to other yeasts [4]. Hence, more and more attention has been focused on the production of chemicals based on *P. pastoris* [5]. However, compared to protein production, the engineering biosynthesis of compounds often requires the integration of multiple heterologous genes and optimization of multiple metabolic pathways. So it is of utmost importance to increase the rate of HR in *P. pastoris* and explore a recyclable selection marker that can be utilized for many rounds of construction to the strain, which is efficiency and timeless.

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Currently, CRISPR/Cas9 systems are widely used for gene editing in *P. pastoris*, including gene mutation, insertion, and deletion [6]. And the CRISPR/Cas9 system has been optimized for increase targeting efficiency and reduce potential off-target effect, including Cas9 gene, guide RNA (gRNA) sequence and their promoters [7]. Stable integration of genes to repair the genome's double-strand breaks (DSBs) in *P. pastoris* is based on homologous recombination (HR) and non-homologous end joining (NHEJ). While HR is an accurate pathway to repair DSBs by using the information from homologous sequences in *Saccharomyces cerevisiae* [8]. However, NHEJ remains the dominant pathway for repairing double-strand breaks (DSB) in *P. pastoris,* which lacks predictability, effectiveness and accuracy. Some studies have demonstrated that deleting the coding gene (*ku70*) of the *P. pastoris* significantly increases HR efficiencies, with gene insertion rates almost reaching 100% [1,8]. And overexpressing the DSB repairing related genes can also increase the positive rate of transformants in *P. pastoris* [9]. Therefore, the top priority is to construct a genomic editing system with highly efficiencies in *P. pastoris*.

In *P. pastoris*, antibiotic resistance screening, such as G418, zeocin, hygromycin, is always used for gene modification [10], which is costly and time-consuming compared to auxotrophic screening. Currently, some selectable marker recycling plasmids are cloned as auxotrophic markers in *P. pastoris,* such as *ARG1*, *MET2, HIS1* [11]*, URA3* and *URA5* [12]. Even though *P. pastoris* GS115 is a histidine auxotrophic host strain, no appropriate agent has been discovered to prevent the growth of *HIS* $^{+}$ cells, thus limiting its use in metabolic engineering for biosynthesis. In contrast, the deletion of uracil biosynthesis genes (*URA3)* has been widely applied for the selectable marker in *S. cerevisiae*. Because *URA3* marker can be removed in one step by 5-Fluoroorotic acid (5-FOA). Unfortunately, uracil auxotrophic strains of *P. pastoris* grow slowly [13,14], even in the presence of uracil, making it challenging to be widely applied in the gene modification. Hence, the key to utilizing *URA3* as the auxotrophic selective marker in *P. pastoris* is to accelerate the growth of uracil auxotroph strains.

To solve the two aforementioned problems, we firstly knocked out the key protein (*ku70*) of NHEJ, and overexpressed HR-related proteins (*RAD52* and *RAD59*), which can increase the positive ratio of HR to about 100% in *P. pastoris*. Then, we knocked out *ura3* to form the uracil auxotrophic strain. Significantly, the uracil transporter (*FUR4*) was integrated into the genome to promote the strain growth rapidly, which facilitated the translocation of uracil from medium. These endeavors resulted in the development of a novel platform in *P. pastoris*, which exhibits high editing efficiencies and a recyclable auxotrophic selective marker, which is identified as HiEE-ReSM system. In the end, we demonstrated the novel platform can be used for the production of inositol using methanol as the sole carbon source. And the titer of inositol achieved 250 mg/L. Overall, our findings present a high efficiency recyclable genetic tool, HiEE-ReSM, for the compound production from C1 substrate in metabolic engineering. And this tool facilitates the application of *P. pastoris* as an extensively versatile cell factory for industrial biosynthesis.

2. Materials and methods

2.1. Strain, plasmids and reagents

All plasmids and strains used in this study are listed in Supplementary Table 1 and Supplementary Table 2. 2 × Phanta® Max Master Mix was purchased from Vazyme (Nanjing, China). PrimeSTAR® HS DNA Polymerase, Premix Taq™ (Ex Taq™ Version 2.0 plus dye) and PrimeSTAR® Max DNA Polymerase were purchased from Takara. Phusion™ High-Fidelity DNA Polymerase, CutSmart buffer (10X), BpiI (BbsI-HF), T4 DNA ligase, 10 mM ATP were purchased from NEB. Hygromycin was purchased from Yeasen (Shanghai, China). DNA cycle pure kit, plasmid purification kit and DNA gel purification kit were purchased from Vazyme. D-sorbitol, DL-dithiothreitol (DTT), ethylene glycol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Myo-inositol was purchased from TCI chemicals (Shanghai, China).

2.2. Strain cultivation

Escherichia coli strain DH5α was used for the construction of plasmids. *E. coli* strains were cultivated in Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) with or without 100 μg/mL of hygromycin or 50 μg/mL ampicillin at 37 °C.

For preparation of competent cells, yeast strains were cultivated in YPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract). Firstly, yeast strains were pre-cultivated in 10 ml tube with 2 ml YPD medium. Then the cells were cultured in 100 ml flasks with 20 ml YPD medium to OD_{600} to 0.6–1.0.

For fermentation of shake flask, the basal medium is minimal medium (Delft), which containing 7.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO4, 0.5 g/L $MgSO_4 \bullet 7H_2O$, 120 mg/L uracil, 10 mg/L histidine, trace metals and vitamin solutions [15,16]. For the dose exploration of uracil and the expression of GFP, 20 g/L glucose was used as the carbon source. For inositol production, 2% (v/v) methanol was used as the sole carbon source.

2.3. Establishing CRISPR–Cas9 system in P. pastoris

The gRNA-Cas9 plasmid was constructed according to the protocol [6]. All primers were listed in Supplementary Table 3. In details, the plasmid pBB3cH_pGAP_23_pLAT1_Cas9 was used as the backbone, which was a gift from Prof. Gao Xiang. The sgRNAs for each target genes were designed and synthesized in Sangon Biotech (Shanghai, China). Then, the sgRNAs were assembled as fusion genes (HH–sgRNA–HDV construct) in an overlap extension (OE) PCR with primers (A-B-C-D, and two sgRNA). The Golden Gate cloning was applied for the plasmid construction. After transformation, the transformants were verified by sequencing. The homology directed gene repair was used in the system, including gene deletion, insertion and replacement.

The preparation of competent cells and DNA transformation were performed followed as the protocol [17]. In detail, the yeast cells were added 9 mL of ice-cold BEDS solution (10 mM bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) DMSO, and 1 M sorbitol) supplemented with 1 mL 1.0 M DTT, and incubated for 5 min at 200 rpm in the 30 °C. After centrifugation at 5000*g* for 5min, the cells were resuspended in BEDS solution. For DNA transformation, condensed electroporation (Bio-Rad Laboratories, Hercules, CA, USA) was used after adding the 1 μg Cas9 plasmid DNA and 500 ng repair fragment into the competent cells using the following parameters (Gene Pulser® II electroporator: cuvette gap, 2.0 mm; charging voltage, 1500 V). For the screening of positive clones from the strains containing hygromycin, YPD medium was supplemented with 200 μg/mL hygromycin. And strains containing *URA3*-based plasmids were cultivated in synthetic complete medium (SC) without uracil. To remove gRNA plasmids from the strain with hygromycin, the correct cells were streaked at least three times on YPD agar plates. For the strain containing plasmid with *URA3* marker, the correct cells were streaked at SC agar plates containing 1.0 mg/mL 5-FOA.

2.4. Single gene deletion and overexpression of HR-related proteins

Protein sequences: *ku70* (Uniprot: F2QWR2), *RAD52* (Uniprot: C4QZT6), *RAD59* (Uniprot: C4R6T9). The *ku70* was replaced with *RAD52* and *RAD59*. The native promoters and terminators were amplified from the genomics DNA of GS115.

The gRNA for *ku70* was chosen from the online website (http://crispr.dbcls.jp). The plasmid, pGSY001, was constructed using CRISPR–Cas9 system. The *Δku70* strain was named as GSY001, and GSY002 strain was integrated with HR-related proteins derived from *P. pastoris* (*RAD52* and

RAD59). All detail information of plasmids and strains were listed in Supplementary Tables S1 and S2.

2.5. Engineering uracil auxotrophic host strain for genetic manipulation

Firstly, the *PpURA3* selectable marker plasmid was constructed as follows. pWY01 was constructed based on the backbone plasmid (pBB3cH_pGAP_23_pLAT1_Cas9) to remove *PpURA3* in genome of GS115. And we also inserted the ampicillin marker into pWY02 for *E.coli* screening. Then, the hygromycin marker was replaced with *PpURA3* (Genbank: AF321098) to construct pWY03 (Table 1). Secondly, the uracil transporter *ScFUR4* (Gene ID: 852309) was integrated into the genome of *URA3* knockout mutant, strain WY01. Finally, the growth curve of strains was used for functional verification of the uracil transporter. Furthermore, the optimal concentration of uracil were detected in SC medium.

2.6. GFP expression at neutral site and the determination of expression level

The plasmid pWY05 was constructed for testing the targeting efficiency of different basal strains. The gRNA sequence for neutral sites II-4 was synthesized according to the literature [9]. The upstream and downstream homologous arms of neutral site, the promoter (GAPp) and the terminator (GAPt) were all amplified from the genomic DNA of *P. pastoris* GS115. Then, the repair fragments were fused with GFP expression cassette, the homologous arms, the promoter and terminators by OE-PCR. In the end, the linearized plasmid pWY05 and repair fragments into the strains by electroporation followed above method. About 3 days later, 12 random transformants were picked to calculate the positive rates and detect the expression intensity of GFP.

For the detection of GFP intensity, strains WY02/03/08/09 with GFP were cultured in 2 ml YPD overnight. Then the cell broth was washed with PBS solution. After adjusting the cell suspension to an OD_{600} of 1.0, the expression intensity of GFP fluorescence was observed by flow cytometry (FACS-celesta). Cultivation and measurements were performed in triplicate. The FITC channel was used to monitor the expression of GFP. Fluorescence intensities normalized against culture OD_{600} (RFU/OD_{600}) were used to indicate the expression intensity of GFP.

Table 1
The CRISPR/Cas9 mediated gene editing system commonly used in *P. pastoris*.

Plasmid backbone	Selection marker	Promoter for Cas9	Promoter for gRNA	Structure for gRNA	Reference
pPICZ	Zeocin	pHTX1	pHTX1	HH-gRNA-HDV	[9]
pPIC3.5K	HIS4/G418	pHTX1	pAOX1/ pTEF1	HH-gRNA-HDV	[1]
pGAPZB	Zeocin	pGAP	pHTX1	HH-gRNA-HDV	[43]
pPIC9	HIS4	pGAP	pHXT1	NA	[44]
pPIC9K	Zeocin	pGAP	pSER	NA	[45]
pPpT4 GAP	Zeocin	pHTX1	pHTX1	HH-gRNA-HDV	[19]
pPpT4 GAP	Zeocin	pHTX1	pHTX1	HH-gRNA-HDV	[46]
pPpT4	HIS4	pHTX1	pHTX1	HH-gRNA-HDV	[47]
pBB3cH	Hygromycin	pLAT1	pGAP	HH-gRNA-HDV	This study
pBB3cH	Uracil	pLAT1	pGAP	HH-gRNA-HDV	This study

2.7. Inositol pathway construction and HPLC analysis

Three inositol synthesis genes inositol-1-monophosphatase *SUHB* (Gene ID: M34828), inositol-3-phosphate synthase *ScINO1*(Gene ID: 853288), *PpINO1* (Gene ID: 8199217) and inositol transporter *ScITR1* (Gene ID: 852108), *PpITR1* (Gene ID: CAY69120) were respectively integrated into II-4 site. The sequence of *SUHB* from *E. coli* was optimized according to the codon usage of *S. cerevisiae*, the sequence information was shown in Supplementary Table 4. Methanol-inducible promoter AOX1p and DAS2p were used for high level expression of proteins. The linker $(GGGGS)_1$ between the protein *INO1* and *SUHB* was desired to enhance the activity of proteins.

After fermentation of WY04 and WY05 for 3 days, 1.5 mL cultural sample was centrifuged at 3000 *g* and the supernatant was filtered through a 0.2 μm filter. Then, the supernatant was analyzed by high-performance liquid chromatography (Agilent Technologies, Agilent 1260 Infinity) equipped with RID (refractive index detector). The column Aminex HPX-87H (300 mm × 7.8 mm, Agilent Technologies) was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

2.8. Statistical analysis

Results were shown as mean ± SD. and data was analyzed by an unpaired two tailed, Student's *t*-test. Statistical significance was set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All data were performed in triplicates.

3. Results

3.1. Construction of the CRISPR-Cas9 system and enhancing HR efficiencies for precise genome editing

As we all know, genome editing *via* the CRISPR-Cas9 system depends on *CAS9* protein and guide RNA (gRNA). The *CAS9* protein is guided by gRNA to create DSB. Normally, there are two ways to activate the DNA repair process in cells, including NHEJ and HR. In NHEJ, DSBs are recognized by the highly conserved *KU70/KU80* heterodimer. The process is uncontrollable and randomized. In contrast, donor DNA fragments can be precisely integrated into the target sites through the process of HR, regulated by multiple HR-related proteins (Fig. 1A). However, NHEJ is the dominant repair mechanism in *P. pastoris*. Previous studies have demonstrated that the deletion of *ku70/ku80* is able to impair NHEJ and improve the HR-rate [8,18,19]. Hence, the *Δku70* strain (GSY001) was firstly constructed using CRISPR–Cas9 system. In order to verify our new system, the GFP as reporter gene was introduced into neutral site II-4 [9] (Fig. 1B). The positive rate and the fluorescence intensity of GFP were used as indicators. Between the WY08 (GS115) and WY09, the positive rate was improved from 75% to 86.1% (Fig. 1C). To further improve the HR efficiency in *P. pastoris*, we introduced the recombination machinery from *S. cerevisiae* [20]. And we over-expressed the HR related protein, *RAD52* and *RAD59,* derived from *P. pastoris* GS115, which are responsible for the DNA repair process during cell growth [9,20]. Our results showed that the positive rate increased to 97% in WY02 compared to WY09 (Fig. 1C). Meanwhile, the GFP expressions were similar among WY02, WY08 and WY09, which indicated that our modifications have no effect on the expression of protein (Fig. 1C). As a result, we built up a highly editing efficiencies (HiEE) CRISPR-Cas9 system in *P. pastoris*.

3.2. Construction of the uracil auxotrophic yeast host strains

In order to construct a novel engineering platform which can be used for multi-step modifications, it is crucial to identify a recyclable selective

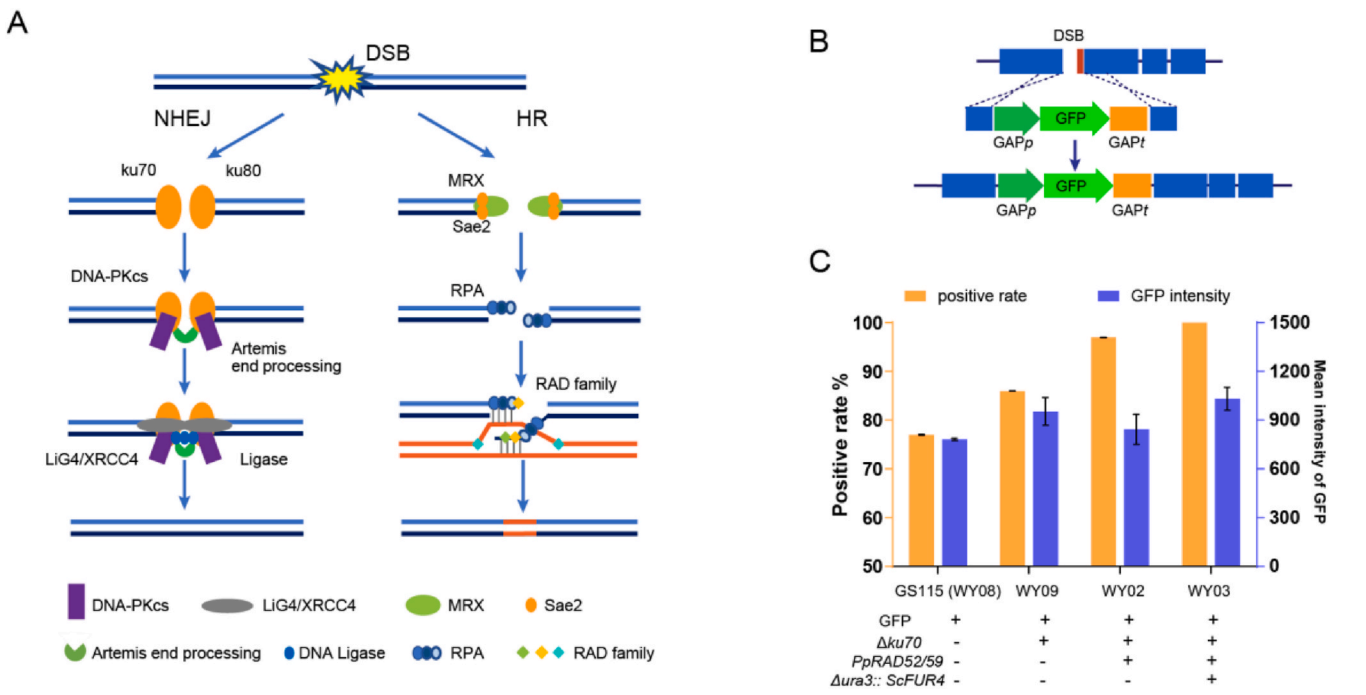

Fig. 1. Engineering recombination machinery to enhance HR efficiencies for precise genome editing.
A. Integral model for the main HR mechanism in *P. pastoris*. In detail, DSBs are generated by the CRISPR-Cas9 system, which can be repaired by enhancing the HR process and repressing NHEJ. The deletion of *ku70* prevented Ku complex formation, eventually decreasing NHEJ activity and repressing NHEJ process. The engineering manipulation of RAD family members, such as *RAD52*, can enhance HR to repair DNA-DSBs for survival. DNA-PKcs: DNA protein kinase catalytic subunit; LiG4: DNA Ligase 4; XRCC4: X-ray repair cross-complementing protein 4; RPA: replication protein A; MRX: Mre11-Rad50-Xrs2;
B. The schematic illustration of GFP expression at genomic neutral site (II-4) in *P. pastoris*. The promoter (GAP*p*) and terminator (GAP*t*) were used for GFP overexpression.
C. The positive rates (%) and the GFP expression were shown in multiple engineered strains.

marker. As we all known, *URA3* can be widely applied for auxotrophic screening of yeast. Meanwhile, 5-FOA is effective and universal as a selective agent. When cells containing an integrated plasmid with *URA3* as a marker are plated onto medium containing 5-FOA, *URA3* (orotidine 5-phosphate decarboxylase) can converts 5-FOA into 5-fluorouracil, which is toxic to growth [21]. Consequently, only *URA3* mutants can be resistant to the 5-FOA, and the strain lacking *URA3* gene or plasmid will survive. So it is convenient and fast to recycle plasmid for the next round of screening compared to the antibiotic screening in *P. pastoris* (Supplementary Fig. 1). Hence, we still choose *URA3* as the screening marker of *P. pastoris*. Firstly, we constructed a plasmid (pWY03) containing both the ampicillin (Amp) maker and *URA3* auxotrophic marker (Fig. 2A). Then, the *PpURA3* was disrupted based on strain (GSY002) to construct the uracil auxotrophic yeast strains. Unfortunately, the correct transformants could not be obtained solely by knocking out the *ura3*. It was not until *FUR4* from *S. cerevisiae* was integrated into the strain GSY002 to form strain WY01 (Fig. 2B) that the desired transformants were finally obtained. The result suggests that the uracil transporter (FUR4) play an important role in the process. Furthermore, we validated the effectiveness of the new system through integrating GFP into the strain WY01, named WY03 (Fig. 2B). The positive rate of WY03 was comparable to the WY02, reaching 100%. And the expression of GFP is slightly higher than WY02 (Fig. 1C). All these results illustrated that the HiEE-ReSM system (strain WY01) can be utilized as the basal strain to multi-step metabolic rewriting.

According to our results, we hypothesized that the growth of yeast depends on the ability of strain to uptake the uracil from the medium. Therefore, the most suitable concentrations of uracil were explored in minimal medium with 2% glucose as carbon source. Apparently, the growth rate of WY01 improved with the increase of uracil concentration ($\leq$90 mg/L). But there was no significant difference when the concentration of uracil exceeded 90 mg/L, such as 120 mg/L and 150 mg/L (Fig. 2C). Thus, the optimal concentration of uracil is 90 mg/L for the growth of uracil auxotrophic yeast. Then, we attempted to increase the copy number of *ScFUR4* in order to improve the growth. Consequently, WY14 and WY15 did not improve the growth of yeast compared to WY01 (Fig. 2D). There was no strong positive correlation between the increasing copy number of *ScFUR4* and the growth rate.

Overall, we constructed a new platform, the HiEE-ReSM system, which can be used for engineering of multi-step metabolic pathways.

3.3. Application of the HiEE-ReSM system for the myo-inositol production in Pichia pastoris

Myo-inositol is a carbocyclic sugar widely distributed in plants, animals and microorganisms. It plays an important role in cell signal transduction in response to various hormones, neurotransmitters, and growth factors, as well as in osmoregulation [22]. Myo-inositol is synthesized from glucose 6-phosphate (G6P) in two steps. First, G6P is isomerized to myo-inositol 1-phosphate by an *INO1*, and then dephosphorylated to free myo-inositol by an *SUHB*. Specially, the transporter of myo-inositol *(ITR1)* is incorporated to export the myo-inositol. *P. pastoris* can grow rapidly on defined medium with methanol as substrate, which is capable to convert methanol to xylulose-5-phosphate (Xu5P) *via* xylulose monophosphate (XuMP) pathway [23]. Then, Xu5P is regenerated into G6P via a cyclic pathway (called the Xu5P cycle) involving reactions of the pentose phosphate pathway (PPP). Based on the metabolic pathway above, myo-inositol can be produced from G6P (Fig. 3A).

In this study, we integrated the *INO1*, *SUHB*, and *ITR1* into the genome of *P. pastoris* GS115. And the strains WY04 and WY05 were constructed by HiEE-ReSM system to produce myo-inositol. The inositol synthase cassettes derived from *S. cerevisiae* and *P. pastoris* GS115, respectively. Previous studies have shown that the GGGGS linker is

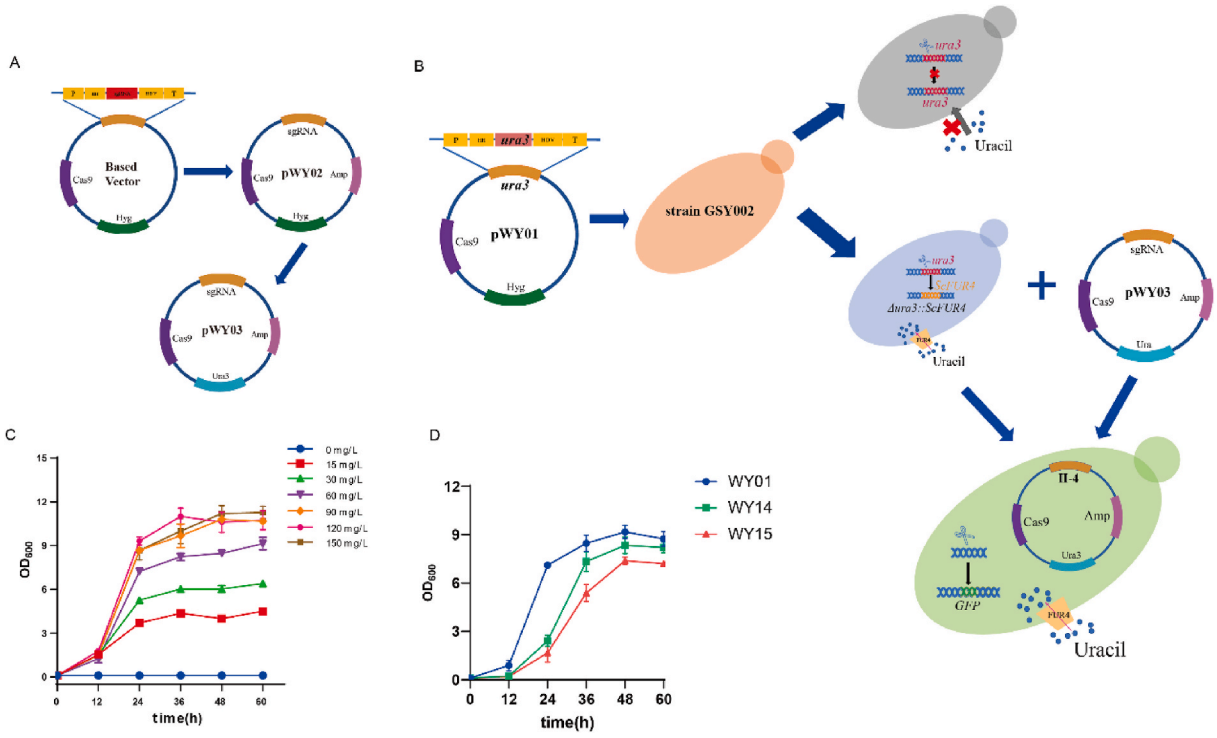

Fig. 2. The growth was improved of the uracil auxotrophic yeast host strains (*Δura3*) via integrating *ScFUR4*.
A. The graphic of the plasmid modification.
B. A schematic diagram illustrating the formation and application of HiEE-ReSM system. The uracil auxotrophic yeast host strains (blue background color) can grow normally with *ScFUR4*. The effectiveness of the system can be validated with auxotrophic yeast containing GFP (green background color).
C. The growth curve of WY01 in minimal medium with different concentrations of uracil and 2% glucose as carbon source.
D. The growth of strain was repressed by increasing the copy number of *ScFUR4*.WY01: only one *ScFUR4*; WY14: 2 copies of *ScFUR4*; WY15: 3 copies of *ScFUR4*.

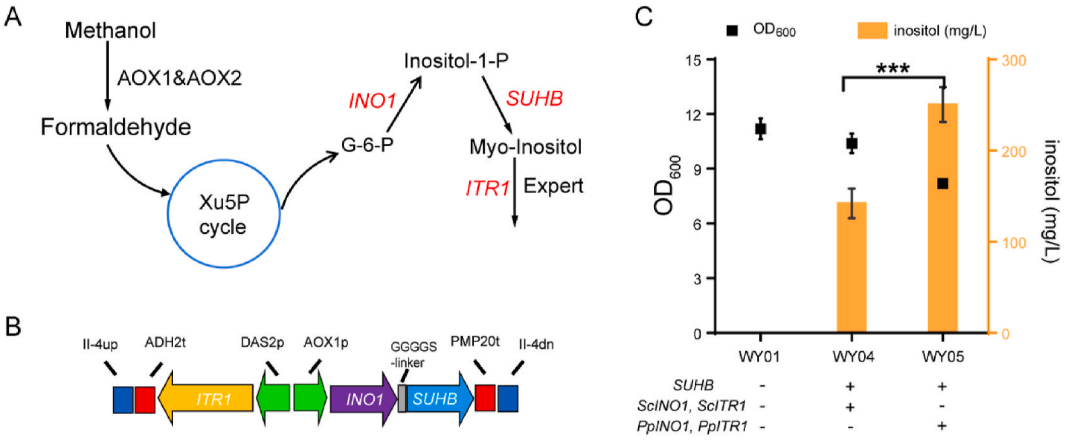

Fig. 3. Application of the HiEE-ReSM system for the myo-inositol production in *P. pastoris*.
A. The biosynthetic pathways of the inositol production in *P. pastoris*.
B. To increasing the inositol production through introducing fusion-expressed proteins.
C. The titer of inositol in strains WY04 and WY05 was measured in minimal medium with 2% methanol as carbon source.

effective in promoting the efficiency of multi-step enzyme reactions [24]. Thus, *SUHB* was fused to the C terminus of *INO1* (Fig. 3B). According to our results, WY04 only produced 142 mg/L inositol derived from methanol (Fig. 3C), while the inositol titer of WY05 was about 250 mg/L, representing an approximately 2-fold increase in production (Fig. 3C). It suggests endogenous *PpINO1* and *PpITR1* are more efficient than exogenous enzymes from *S. cerevisiae*. Hence, the production of myo-inositol from methanol demonstrated the great potential of our HiEE-ReSM system as a chassis for methanol-based bio-refinery.

4. Discussion

As a methylotrophic budding yeast, *P. pastoris* has been used for heterologous protein production due to its GRAS (Generally regarded as safe) status, rapid growth rate and ability for high cell density fermentation [25]. Recently, *P. pastoris* has gained considerable interest as a host for metabolic engineering, particularly for the production of value-added products using glucose or glycerol as substrate, such as myo-inositol [22], malic acid [26], lycopene [27]. Notably, *P. pastoris* can grow well on defined chemical medium with methanol as the sole

carbon source, which can promote the biotransformation of methanol toward value-added chemicals, such as C4-dicarboxylic acids [28], fatty acid derivatives [29], and lovastatin [30]. Meanwhile, with the breakthrough of the technology for methanol synthesis, the price of methanol has experienced a constant decline [31]. However, the low efficiency rate of HR and the lack of handy recyclable auxotrophic selective marker make it challenging to expand its product portfolio and enhance the methanol biosynthesis. Therefore, it has the high industrial application potential for synthesizing complex chemicals derived from methanol. It is urgent to develop an efficient and convenient method for gene editing in *P. pastoris*. In our study, we established a high efficiency recyclable genetic tool in *P. pastoris*, HiEE-ReSM system, which can increase the rate of HR in this yeast and construct the uracil auxotrophic yeast host strain. These advancements may further promote its application as a cell factory for the biosynthesis of valuable products from methanol.

The CRISPR–Cas9 system has been developed as a powerful tool for gene editing successfully applied in *S. cerevisiae* [32], *O. polymorpha* [33], *P. pastoris* [9]. Compared to the high HR rate observed in *S. cerevisiae*, the relatively high NHEJ rates in non-conventional yeasts limit the precise manipulation. According to previous studies, the deletion of *ku70/ku80* significantly improved the positive rate of gene deletion in *P. pastoris* [1,8,19]. Firstly, we knocked out the key protein (*ku70*) of NHEJ, and the positive rate achieved around 86% using PCR fragments with homology arms of a universal length (500–1000 bp) (Fig. 1C). This result is slightly lower than the previously reported HR frequencies, where 100% positive rate was achieved using 650–1350 bp homologous flanking regions on each side of the integration cassette [8]. We speculated that the variation in HR rates may be due to the differences in screening markers and gRNA-Cas9 plasmids. In order to improve further the HR rates in our system, we followed the HR machinery in *S. cerevisiae,* which achieved nearly 100% targeted repair even with short homology arms [34]. In *S. cerevisiae*, after the DSB is recognized, the single-stranded DNA is generated by *SAE2* [35]. Afterwards, the strand invasion and exchange are accomplished by a series of proteins from *RAD* family, including *RAD51*, *RAD52* [36], *RAD59* [37]. Furthermore, the much higher positive rates can be obtained by overexpressing endogenous *PpRAD52* in *P. pastoris* [9]. Therefore, we anticipated that the HR rates will be improved by overexpressing the endogenous *PpRAD52* and *PpRAD59*. Our results confirm that the hypothesis was correct, as the positive rate improved to nearly 100% in our system (Fig. 1C). Meanwhile, our findings indicated that the genomic modification in *P. pastoris* have no effect on the protein expression, such as the intensity of GFP, which is critical for the insertion of numerous exogenous gene to biosynthesize the chemicals in the future. So far, we have established a highly editing efficiencies (HiEE) system.

Besides these efficient and precise genome engineering tools, our research also focus on finding a recyclable screening marker, which can increase the efficiency and reduce time for multi-step genome editing. To address this problem, some selectable marker recycling plasmids are currently in use [10], including auxotrophic screening markers [11,12] and antibiotic resistance screening markers [38,39]. Compared to antibiotic resistance screening, auxotrophic screening is more economical and convenient. Several genes have been cloned as auxotrophic marker in *P. pastoris* [11,14], especially as *URA* [12], which have been widely used as the counter-selectable markers in many microorganisms [15,40,41] because it can be easily removed in one step by 5-FOA. However, the application of the uracil auxotrophic strain of *P. pastoris* in engineering transformation remains limited. For one thing, there is a broad range of positive rates when only *ura3 or ura5 was* knock out in *P. pastoris*, ranging from 44% to 90% [11], which make it challenging to obtain the correct transformants. Now, a HiEE system with highly HR rates has been developed, which may improve the HR rates in *Δura3* strain based on GSY002 strain. And our results support the conjecture (Fig. 1C). For another, the major drawback of deleting *ura3* or *ura5* is the growth defect that occurs. The *ura3* and *ura5* auxotrophs of *P. pastoris* have a significantly reduced growth rate of 1.75 OD/h in YPD medium, while the growth rate is 3.3OD/h in YPD medium of wild-type *P. pastoris* [14]. When plated onto SC agar plates supplemented with 5-FOA, the strain exhibited growth for an additional 7 days [12]. And in our results, the *Δura3* strain exhibited very little growth, as there were almost no transformants. Furthermore, previous studies demonstrated that the growth rate can be restored through re-transforming the *URA3*--containing vector into the uracil strains. The results showed that the slow growth rate was due to the limited ability of *P. pastoris* to transport uracil into the cell efficiently [14]. We speculated the growth rate of uracil auxotrophic yeast can be recovered by integrating the uracil transporter. To test this, *FUR4* from *S. cerevisiae* [42] was integrated, which can uptake the uracil from medium. And it was able to obtain the correct transformants (*Δura3* strain, WY01) within 4 days (Fig. 2B). Furthermore, the positive rate of WY03,based on *Δura3* strain WY01, also reached 100% (Fig. 1C). Surprisingly, we found that the growth rate of the strain WY01 can be increased through adding different concentrations of uracil, and the optimal concentration of uracil was determined to be 90 mg/L (Fig. 2C). However, the growth rate can not be further improved via increasing the copy number of *ScFUR4* (Fig. 2D). Now, we have established a high efficiency and recyclable auxotrophic genetic tool in *P. pastoris,* HiEE-ReSM system.

In the end, we verified the new platform can be used to the production of inositol using methanol as the sole carbon source, and the titer of inositol achieved 250 mg/L (Fig. 3C). In the future, more compounds can be synthesized with this platform in *P. pastoris*. In summary, our findings provide a high efficiency and auxotrophic recyclable genetic tool, HiEE-ReSM, that can grow normally as the uracil auxotrophic yeast. It facilitates the application of *P. pastoris* as an extensively cell factory for industrial C1 biosynthesis.

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CRediT authorship contribution statement

Xiang Wang: performed the experiments, Formal analysis, Writing – original draft. **Yi Li:** assisted with the plasmid construction of the neutral site. **Zhehao Jin:** assisted with article revision. **Xiangjian Liu:** assisted with the experiment of DNA electroporation transformation. **Xiang Gao:** provided us with engineered plasmids and genetic modification technology. **Shuyuan Guo:** performed partial experiments, Formal analysis, Writing – original draft. **Tao Yu:** Writing – original draft.

Declaration of competing interest

The authors declare that they do not have any financial or commercial conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2023.06.003.

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