

Construction of Three Auxotrophic *Pichia pastoris* Strains

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Abstract:

In recent years, non-conventional yeasts with unique stress tolerance and substrate utilization capacity have attracted increasing attention. Among them, *Pichia pastoris* has emerged as a well-established host for recombinant protein production owing to its compact 9.4 Mb genome, ability to utilize methanol, and excellent high-cell-density fermentation performance. Beyond recombinant proteins, *P. pastoris* has been widely extended to the biosynthesis of natural products and basic biological research. Its major advantages include stable genomic integration, efficient secretion and post-translational modification of heterologous proteins, low-cost cultivation, and FDA-recognized biosafety, which make it particularly suitable for large-scale industrial applications.

Despite these advantages, the lack of versatile genetic selection markers remains a critical bottleneck in *P. pastoris*. Conventional antibiotic-based selection suffers from several drawbacks, such as interference with methanol metabolism, induction of false-positive resistant mutants, increased cost and purification burden, potential biosafety concerns, and limited applicability in multiple rounds of genome editing. Inspired by the well-established auxotrophic strategy in *Saccharomyces cerevisiae*, we sought to expand the genetic toolkit of *P. pastoris* by constructing auxotrophic strains. Considering that *P. pastoris* predominantly repairs double-strand breaks via non-homologous end joining (NHEJ), we designed specific gRNAs to guide Cas9-mediated cleavage and successfully disrupted three key auxotrophic marker genes (*his4*, *arg*, and *met*). This enabled the establishment of triple-auxotrophic strains.

The resulting strains provide multiple, orthogonal selection loci that can be independently recycled, significantly reducing compatibility conflicts in multigene pathway assembly and lowering the overall cost of strain engineering. More importantly, these strains lay the foundation for iterative, large-scale development of *P. pastoris* as a versatile cell factory for synthetic biology and industrial biotechnology.

Key Word: *Pichia pastoris*; CRISPR/Cas9; auxotrophic marker; genome editing; non-homologous end joining.

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I. Introduction

Yeasts have long been exploited as versatile cell factories for the production of proteins, chemicals, and biofuels. *Saccharomyces cerevisiae* has served as the workhorse for producing many industrially relevant biochemicals and biofuels^[1]. More recently, several non-conventional yeasts have attracted considerable interest as alternative chassis cells for the synthesis of fine chemicals and recombinant proteins. These yeasts possess distinctive traits, such as enhanced tolerance to inhibitors and low pH, which confer unique advantages in specific bioprocesses^[2]. Among them, the methylotrophic yeast *Pichia pastoris* (reclassified as *Komagataella phaffii*) has emerged as one of the most established platforms for recombinant protein production^[3].

P. pastoris is an ascomycetous yeast with a genome of approximately 9.4 Mb distributed across four chromosomes and encoding ~5,200 genes^[4]. It harbors one of the most efficient methanol assimilation pathways known, allowing methanol to serve as its sole carbon and energy source. Since the 1990s, it has been widely recognized as a preferred host for recombinant protein expression and has more recently been applied to the biosynthesis of non-protein products such as natural compounds^[5]. In addition, *P. pastoris* is increasingly used as a model organism in biomedical and fundamental cell biology research^[6].

The advantages of *P. pastoris* include thermotolerance, osmotolerance, high-cell-density cultivation, and the ability to produce recombinant proteins at high levels, which has led to its adoption in pharmaceutical, feed, and food industries^[7]. Its system enables stable genomic integration, efficient secretion of properly glycosylated proteins, and cultivation under cost-effective conditions. Moreover, it combines high productivity with preservation of product bioactivity and is well suited for industrial-scale applications^[8]. Products generated from *P. pastoris* have already undergone evaluation by the U.S. Food and Drug Administration (FDA), underscoring its biosafety and regulatory acceptance. Importantly, its methanol assimilation pathway is phylogenetically distinct from those of other organisms, further enhancing its value for both applied and basic research.

Compared with *S. cerevisiae*, which primarily relies on conventional sugars, *P. pastoris* efficiently utilizes methanol, glycerol, glucose, and other carbon sources, thereby broadening the range of inexpensive substrates available for industrial fermentation. Despite these advantages, the lack of sufficient selection markers in wild-type *P. pastoris* remains a bottleneck for genetic engineering. Both plasmid-based and genome-integrated heterologous constructs require reliable selection, yet the current marker repertoire is very limited.

Antibiotics, though often used as selection markers, present multiple drawbacks. They can interfere with methanol metabolism by impairing mitochondrial function and lowering the induction efficiency of the AOX1 promoter. They also promote spontaneous resistant mutants, leading to high false-positive rates. In addition, antibiotics are costly, require additional purification to remove residual compounds, and raise biosafety concerns due to potential environmental dissemination of resistance genes. Most critically, antibiotic-based selection is unsuitable for iterative genetic engineering, as resistance plasmids are difficult to eliminate after multiple passages without selective pressure. These limitations severely constrain large-scale and multi-step engineering strategies.

Inspired by the auxotrophic strain BY4741 of *S. cerevisiae*, we aimed to construct a *P. pastoris* strain harboring multiple auxotrophic mutations. Such nutritional markers provide independent selection loci, facilitate the parallel assembly of multiple expression modules, minimize compatibility conflicts, and reduce production costs.

Unlike *S. cerevisiae*, where homologous recombination predominates, *P. pastoris* repairs double-strand breaks (DSBs) primarily through non-homologous end joining (NHEJ), which complicates precise genome editing and seamless gene knockouts [3]. To address this challenge, we adapted the CRISPR/Cas9 system for *P. pastoris* by exploiting gRNA-directed Cas9 cleavage in conjunction with the NHEJ pathway. Using the wild-type GS115 strain as the starting point, we targeted two nutritional marker genes, *arg* [9] and *met* [10–11], for disruption. Experimental validation demonstrated efficient cumulative knockouts, establishing the foundation for constructing multi-auxotrophic strains and enabling more flexible, iterative engineering of *P. pastoris* as a robust microbial cell factory.

II. Material And Methods

2.1 Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1.

Table 1: Strains and plasmids used in this study.

Strain/Plasmid	Phenotype/Characteristics	Source
<i>P. pastoris</i> GS115	<i>his4</i> Mut ⁺	Our lab
<i>P. pastoris</i> GS115Δ <i>arg</i>	<i>his4</i> Δ <i>arg</i> Mut ⁺	This study
<i>P. pastoris</i> GS115Δ <i>arg</i> Δ <i>met</i>	<i>his4</i> Δ <i>arg</i> Δ <i>met</i> Mut ⁺	This study
pCas9-gRNA	CRISPR-Cas9-assisted genome editing in <i>P. pastoris</i> GS115, <i>Bleo</i> ^R	From Dr. Zhou [3]
pCas9-Δ <i>arg</i>	sgRNA- <i>arg</i>	This study
pCas9-Δ <i>met</i>	sgRNA- <i>met</i>	This study

2.2 Experimental Methods

2.2.1 gRNA Design and Plasmid Construction

Target genes were selected for knockout, and gRNA sequences were designed using the CHOPCHOP online tool. Since gene disruption in this study relied on gRNA-guided NHEJ repair, target sites were preferentially selected near the 5' end of the coding sequence. The primers used are listed in Table 2.

Based on the pCas9-gRNA vector reported by Zhou et al. [3], gene-specific N20 sequences were incorporated into the plasmid using primers listed in Table 2. Overlap PCR was performed to amplify the gRNA fragments, which were subsequently cloned into the linearized pCas9-gRNA backbone via seamless cloning (In-Fusion), thereby replacing the original N20 sequence.

2.2.2 Transformation

Yeast cells were cultured overnight in 5 mL YPD medium and then inoculated into 50 mL fresh YPD. Cultures were incubated at 30 °C with shaking until OD₆₀₀ reached 0.8–1.0 (approximately 4–5 h). Cells were harvested by centrifugation at 500 × g for 5 min at room temperature and resuspended in 9 mL ice-cold BEDS solution [12] supplemented with 1 mL 1.0 M dithiothreitol (DTT). The suspension was incubated at 30 °C, 100 rpm for 5 min, centrifuged again at 500 × g for 5 min, and finally resuspended in 1 mL BEDS solution without DTT.

Table 2: Primer used in this study.

Primer	Primer sequence (5'-3')
$\Delta arg4$ -1-F	CATCGTTTCGACTAGTTGTTGTAGTTT TAATATAGTTTGAGTATGAGATGGA
$\Delta arg4$ -1-R	TAGTTCGACGAGCTTACTCGTTTCGTC CTCACGGACTCATCAGGAACATTTG ATTGT
$\Delta arg4$ -2-F	AAGCTCGTCGAACTAAGTGAGATTCA TCGGTTTATAGAGCTAGAAATAGCAAG TAAAA
$\Delta arg4$ -2-R	CATAATCAGCACTAGGTACCGCACAA ACGAA
Δmet 2-1-F	CATCGTTTCGACTAGTTGTTGTAGTTT TAATATAGTTTGAGTATGAGATGGA
Δmet 2-1-R	GAGATGGACGAGCTTACTCGTTTCGTC CTCACGGACTCATCAGCATCTCTTTGA TTTGT
Δmet 2-2-F	AAGCTCGTCCATCTCTGACTGATACAA GGGTTTATAGAGCTAGAAATAGCAAGT TAAAA
Δmet 2-2-R	CATAATCAGCACTAGGTACCGCACAA ACGAA

Approximately 4 μ L of plasmid DNA was mixed with 40 μ L competent cells in an electroporation cuvette and incubated on ice for 5 min. Electroporation was performed under the following conditions: 1500 V, 200 μ F, 50 μ F (2.0 mm cuvette). Immediately after the pulse, the cells were recovered in 1 mL ice-cold 1.0 M sorbitol and spread onto selective media.

2.2.3 Screening of Transformants

Single colonies appearing on selective media were replica-plated onto media with or without the corresponding auxotrophic marker. Colonies able to grow on media supplemented with the deleted amino acid, but not on media lacking it, were identified as successful knockout strains.

III. Result

3.1 Single Knockout of *arg* in *P. pastoris* GS115

CRISPR-mediated genome editing was employed to disrupt the *arg* gene in *P. pastoris* GS115. Following double-strand break induction, NHEJ repair caused base insertions or deletions, leading to frameshift mutations and loss of gene function.

Transformants harboring the *arg*-targeting gRNA plasmid exhibited an auxotrophic phenotype. Specifically, GS115 Δarg strains could not grow on MD+His plates but regained growth on MD+His+Arg plates, confirming the successful knockout of the *arg* gene (Figure 1).

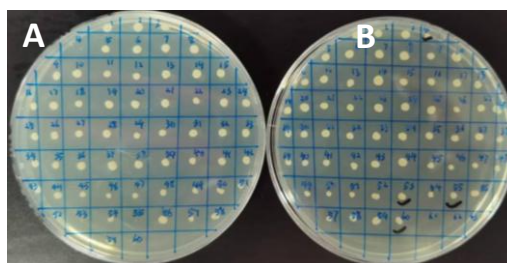


Figure 1: Knockout of *arg* gene in GS115 using CRISPR-Cas9 and NHEJ. (A) Growth of GS115 Δarg on MD+His medium. (B) Growth of GS115 Δarg on MD+His+Arg medium. Colonies 4, 52, 53, 55, and 60 were confirmed as successful knockouts.

3.2 Sequential Knockout of *met* in GS115 Δarg

After plasmid curing through serial passaging under nonselective conditions, GS115 Δarg strains were retransformed with the Δmet -gRNA plasmid. Colonies were tested on MD+His+Arg+Met and MD+His+Arg plates. Those that grew only on MD+His+Arg+Met plates were identified as double knockouts (Figure 2).

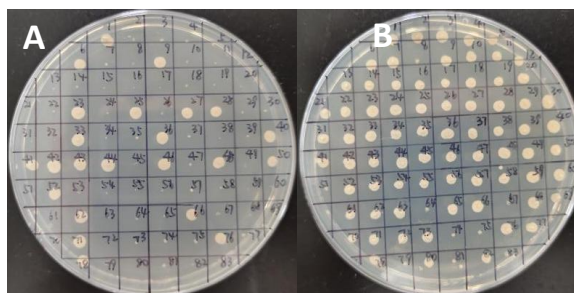


Figure 2: Sequential knockout of *met* in GS115 Δ *arg* background.(A) Growth of GS115 Δ *arg* Δ *met* on MD+His+Arg medium.(B) Growth of GS115 Δ *arg* Δ *met* on MD+His+Arg+Met medium.Colonies 2, 3, 4, and 5 were confirmed as double knockouts.

In addition, we observed that multi-auxotrophic strains exhibited slower growth, with triple amino acid auxotrophic mutants growing particularly poorly. Future work may require adaptive evolution or metabolic engineering to improve their growth phenotypes.

IV. Conclusion

Non-conventional yeasts are increasingly recognized as promising chassis for synthetic biology, owing to their Crabtree-negative metabolism, thermotolerance, and broad substrate utilization [13]. Among them, *Pichia pastoris*, designated as “Generally Recognized as Safe (GRAS)” by the FDA, has emerged as a preferred platform for industrial recombinant protein production due to its exceptional protein folding and secretion capacity.

Here, we engineered a *P. pastoris* strain harboring triple auxotrophic mutations (*his4* Δ , *arg4* Δ , and *met2* Δ) by employing the CRISPR/Cas9 system coupled with the native non-homologous end joining (NHEJ) pathway. This multi-auxotrophic chassis provides orthogonal selection markers that allow simultaneous pathway assembly and modular regulation, thereby facilitating the efficient biosynthesis of complex metabolites such as β -carotene. Moreover, these auxotrophic markers can be flexibly repurposed to introduce other genome engineering tools (e.g., SCRaMbLED), greatly enhancing the genetic versatility of the strain. Collectively, this work establishes an antibiotic-free selection strategy that expands the capacity of *P. pastoris* as a universal cell factory for the scalable production of multi-component natural products.

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