Enhancement of the Gene Targeting Efficiency of Non-Conventional Yeasts by Increasing Genetic Redundancy

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Abstract

In contrast to model yeasts, gene targeting efficiencies of non-conventional yeasts are usually low, which greatly limits the research and applications of these organisms. In this study, we aimed to enhance the gene targeting efficiency of non-conventional yeasts by improving the fitness of mutant strains, particularly by increasing the genetic redundancy of host cells. To demonstrate this process, OCH1 gene deletion in Pichia pastoris was performed. Extra copies of the OCH1 gene on a helper plasmid were provided for the P. pastoris GS115 strain before the native OCH1 gene in the genomic DNA was knocked out. The redundancy in OCH1 gene significantly eliminated the growth defects of the och1 mutant and increased the deletion efficiency of the OCH1 gene by two orders of magnitude with the same length of homologous flanks. The same strategy was used to delete the KU70 and SGS1 genes. The targeting efficiencies of KU70 and SGS1 were increased by 1- and 23-fold, respectively. Therefore, this study provided an efficient strategy for the deletion of “stubborn” genes in non-conventional yeasts. This study further showed that cellular fitness is potentially an important factor that can limit the efficiency of gene targeting.

Introduction

Gene targeting (e.g., targeted gene replacement) is one of the main molecular tools used in yeast science, which helps in understanding gene functions and interactions as well as cellular and molecular processes in yeasts [1]. Model yeasts, such as Saccharomyces cerevisiae and Schizosaccharomyces pombe, have very efficient gene targeting systems. For instance, disruption cassettes with short flanking regions that range from 30 bp to 50 bp can integrate with high efficiencies via homologous integration at the correct genomic locus (routinely >70%) in S. cerevisiae [2]. In contrast, the gene targeting efficiencies of various “non-conventional” yeasts [3,4], such as Pichia pastoris, Hansenula polymorpha, Yarrowia lipolytica, Pichia stipitis and Kluiveromyces lactis can be extremely low (usually <1%) with the same length of flanking regions. Accordingly, homologous arms varying from 200 bp to approximately 2000 bp are usually required to ensure efficient gene replacement in non-conventional yeasts [1]. Nevertheless, for some “stubborn” genes, the probability of obtaining the desired gene replacement event is so low that transformation and/or screening procedures have to be iteratively performed, which is laborious and time-consuming. Therefore, the low targeting efficiencies of non-conventional yeasts greatly limits the researches and applications of these industrially important microorganisms.

To improve the targeting efficiencies of non-conventional yeasts, molecular mechanisms of gene targeting should be understood. Yeasts have the homologous recombination (HR) pathway and the non-homologous end-joining (NHEJ) pathway. The HR pathway, which depends on the Rad52 epistatic group, is responsible for the targeted integration of DNA [5]. Integration can also be mediated randomly via the NHEJ pathway, which depends on the Ku70/Ku80 protein complex [6]. When foreign DNAs are transformed into cells, they are competed by these two recombinant pathways. Therefore, efficient gene targeting is determined by the relative strength of the HR pathway compared with that of the NHEJ pathway. The bias, which favors the NHEJ pathway in non-conventional yeasts, determines their low targeting efficiencies [1].

However, NHEJ pathway bias is not the only factor that contributes to the low targeting efficiency of non-conventional yeasts. The efficiency of homologous integration in strains with the same genetic background can be very locus specific. For example, the disruption of ARG1, ARG2, ARG3, HIS1, HIS2, HIS3, and HIS6 in P. pastoris GS115 strain with flanking arms that range from 200 bp to 900 bp is considerably efficient at frequencies of 44% to 90% [7]. However, the deletion of PEM [8] and OCH1 [9] from the same strain that contains both flanking arms that were longer than 200 bp to 900 bp occurred at a frequency of <1%. To date, this locus specific phenomenon is not well understood. One explanation is the presence of “hotspot” regions for yeast genome, in which the underlying mechanism still remains unclear [10]. Another, which is often overlooked in previous studies, may be the loss of function effect. Several non-conventional yeasts such as P. pastoris, H. polymorpha, Y. lipolytica, and P. stipitis are predominantly haploid.
Thus, the knockout of genes with important physiological functions often means great loss of cellular fitness, which would lead to a delayed or failed appearance of correct disruptants. Therefore, the calculated frequency of correct gene targeting by colony counting is likely lower than the actual frequency of gene targeting events that happen genetically because of the unformed colonies. To validate this assumption as well as provide an efficient solution to delete “stubborn” genes in haploid non-conventional yeasts, in this study, we proposed a new strategy (Fig. 1) for enhancement of gene targeting efficiency by improving cellular fitness of mutant cells, specifically by increasing the genetic redundancy of host cells. To achieve this goal, the targeted gene was cloned into an expression vector (helper plasmid), and transformed into yeast cells to generate the transition host. Targeted gene disruption was then applied in the transition host by transformation with the disruption plasmid. After gene deletion in the genome was successfully validated, the helper plasmid can be easily removed.

>P. pastoris<, a methylotrophic yeast, was used as a model in this study for two reasons: 1) the efficiency of targeted gene replacement in <P. pastoris> is very low, which is estimated to occur at a frequency of 0.1% when the total length of the target fragments is 500 bp [13]; and 2) <P. pastoris> is of great industrial importance. It is by far one of the most often used yeast species in the production of recombinant proteins [14]. The <OCH1> gene of <P. pastoris> was chosen as an illustrative example because its deletion procedure by double homologous recombination is notoriously inefficient [9,15]. To demonstrate the effectiveness of this strategy,

![Figure 1. Schematic representation of the new strategy for efficient gene targeting.](image-url)

**Table 1.** Strains and plasmids used in this work.

| Strains | Characteristics | Reference |
|---------|-----------------|-----------|
| E.coli DH5α | General cloning host strain | Takara |
| P. pastoris GS115 | hisα mutant | Invitrogen |
| GS-tranOCH1 | GS115 carrying pGKARSmazf-OCH1 | This work |
| GS-tranSGS1 | GS115 carrying pGKARSmazf-SGS1 | This work |
| GS-tranKU70 | GS115 carrying pGKARSmazf-KU70 | This work |
| KO24# | Reconstructed by single crossover | This work |
| GS-tranOCH1-ΔOCH1 | och1 mutant carrying pGKARSmazf-OCH1 | This work |
| GS-ΔOCH1 | och1 mutant | This work |

**Plasmids**

| Plasmids | Characteristics | Reference |
|----------|-----------------|-----------|
| pUG6 | Base vector for constructing pZeoloxp vector | Invitrogen |
| pUG-zeoloxp | Vector for constructing pZeoloxp | This work |
| pZeoloxp | Vector for constructing disruption plasmids | This work |
| pZeoloxp-OCH1 | pZeoloxp based vector for deletion of OCH1 | This work |
| pZeoloxp-SGS1 | pZeoloxp based vector for deletion of SGS1 | This work |
| pZeoloxp-KU70 | pZeoloxp based vector for deletion of KU70 | This work |
| pGAPZB | Vector for constitutive protein expression | Invitrogen |
| pPIC9K | Vector used for amplification of Kan<sup>R</sup> cassette | Invitrogen |
| pGAPKB | pGAPZB based vector replacing Zeo<sup>R</sup> into Kan<sup>R</sup> | This work |
| pGKARS | pGAPKB based vector carrying an PARS2 | This work |
| pPICZA | Vector for amplification of Zeo<sup>R</sup>, Amp<sup>R</sup> and Ori | Invitrogen |
| pGKARS-OCH1 | Vector for constitutive expression of ORF OCH1 | This work |
| pGKARS-SGS1 | Vector for constitutive expression of ORF SGS1 | This work |
| pGKARS-KU70 | Vector for constitutive expression of ORF KU70 | This work |
| pGKARS-mazf-OCH1 | pGKARS-OCH1 based vector carrying a mazf gene | This work |
| pGKARS-mazf-SGS1 | pGKARS-SGS1 based vector carrying a mazf gene | This work |
| pGKARS-mazf-KU70 | pGKARS-KU70 based vector carrying a mazf gene | This work |

**Abbreviations:** Amp<sup>R</sup>, ampicillin resistance; Kan<sup>R</sup>, kanamycin resistance; Zeo<sup>R</sup>, zeocin resistance.

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the deletion of two other genes, namely, SGS1 and KU70, was also assessed using the same method.

Materials and Methods

Strains, Plasmids, and Oligonucleotides

A complete list of strains and plasmids is presented in Table 1. Oligonucleotide synthesis (Table S1) and DNA sequencing were performed at the Shanghai Sangon Biological Engineering Technology and Service Co. (Beijing, China).

Media and Growth Conditions

*Escherichia coli* DH5α strain was grown at 37°C in LLB broth (10 g L⁻¹ of tryptone, 5 g L⁻¹ of yeast extract, 5 g L⁻¹ of NaCl) or LB medium (10 g L⁻¹ of tryptone, 5 g L⁻¹ of yeast extract, 10 g L⁻¹ of NaCl). *P. pastoris* was cultivated aerobically at 30°C in yeast extract peptone dextrose (YPD) medium (10 g L⁻¹ of yeast extract, 20 g L⁻¹ of peptone, 20 g L⁻¹ of glucose). Antibiotics were added at the following concentrations: 100 mg L⁻¹ of ampicillin, 50 mg L⁻¹ of kanamycin, and 25 mg L⁻¹ of Zeocin (Invitrogen) for *E. coli*; 500 mg L⁻¹ of geneticin (G418, Invitrogen) and 50 mg L⁻¹ of Zeocin for *P. pastoris*.

Construction of Disruption Plasmids

pZeolOxp, the base vector used to construct all of the other disruption plasmids, was constructed based on the following procedures. Zeocin resistance cassette was amplified from pPICZA with P_{rev1}/P_{rev2}, digested with SacI and BglII, and cloned into pUG6 to replace the kanMX cassette between the loxP sites, thereby producing the pUG-zeoloxp plasmid. The origin of replication (ori), which was amplified from pPICZA by polymerase chain reaction (PCR) with P_{ori1}/P_{ori2} primer pair and digested with SalI, was inserted into the XhoI site of pUG-zeoloxp. The DNA fragment with ori and ampicillin resistance gene was then excised through NotI digestion and religation.

To construct the disruption plasmid for OCH1 deletion, the upstream and downstream homologous regions of OCH1 were initially amplified through PCR from *P. pastoris* GS115 strain by using two primer pairs, namely, OCH1-N3/OCH1-N5, and OCH1-C3/OCH1-C5. The two fragments were cut with SpeI, NotI, and PstI, ligated into pZeolOxp which was cut with SpeI and PstI, thereby generating pZeolOxp-OCH1 (Fig. 2). The disruption plasmids used to delete the SGS1 and KU70 genes, namely, pZeolOxp-SGS1 and pZeolOxp-KU70 were constructed following...
to facilitate the removal of pGKARS-OCH1 as necessary. To KU70, respectively.

Construction of the Episomal Plasmids

pGAPZB and the kanamycin resistance gene, amplified from pPIC9K with Ppam1/Pmazf, were cut using NcoI/Stul and ligated by the T4 DNA ligase to replace the original Zeocin-resistant gene, thereby generating the pGAPKB plasmid. PARS2, an autonomous replication sequence of P. pastoris [16], was amplified from the genomic DNA of GS115 strain with Ppars2F/Ppars2R, cut with BamHI and BglII, and inserted into the BglII site of pGAPKB to generate the episomal expression plasmid, pGKARS. To express OCH1, SGS1, and KU70 in P. pastoris with pGKARS, their encoding genes were amplified from the GS115 genome by using their respective primer pairs (Table S1). PCR fragments were cut using EcoRI and NotI, and then ligated into the corresponding pGKARS sites to produce the episomal expression plasmids, namely, pGKARS-OCH1, pGKARS-SGS1 and pGKARS-KU70, respectively.

An inducible mazf cassette was inserted into pGKARS-OCH1 to facilitate the removal of pGKARS-OCH1 as necessary. To construct the mazf cassette, the mazf gene was amplified from E. coli by using the Pmazf1/Pmazf2 primer pair, digested with EcoRI and NotI, and ligated into the corresponding pPICZA sites to place the mazf gene under the control of the alcohol oxidase (AOX1) promoter. The mazf cassette was then excised from the resulting plasmid by BglII and BamHI digestion and inserted into the BglII site of pGKARS-OCH1, thereby generating the pGKARSmazf-OCH1 plasmid (Fig. 3).

Competent P. pastoris Cell Preparation and Transformation

The competent cells were prepared based on a revised version of a previously described method [17] to achieve a highly efficient transformation of P. pastoris. Briefly, a fresh single clone was inoculated into 100 ml YPD medium and grown overnight at 30°C by shaking at 200 rpm until the cell density reached an OD600 of 1~2. The cells were pelleted and resuspended at room temperature for 30 min in 8 ml of 100 mM LiAc, 10 mM diethiothreitol, 0.6 M sorbitol, and 10 mM Tris-hydrochloride at pH 7.5. The resulting cells were then washed thrice with 2 ml to 3 ml of 1 M ice-cold sorbitol. Finally, the cells were suspended in 1 M ice-cold sorbitol and transferred to 1.5 ml microcentrifuge tubes with an aliquot of 80 μl. P. pastoris GS115 strain was transformed by electroporation according to the protocols outlined by Invitrogen.

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Direct Gene Disruption and Verification

The disruption plasmids (pZeoloxp-OCH1, pZeoloxp-SGS1, or pZeoloxp-KU70) were linearized by NotI and transformed into P. pastoris GS115 strain. The cells were then spread on YPD plate that contains 50 mg L⁻¹ of Zeocin to screen the Zeocin-resistant transformants.

The transformants were further analyzed by colony PCR to verify whether they contained the correct chromosomal integrations of the gene disruption cassette. The parental strains were used as the control group. Two primer pairs (Fig. 4A), P9 (located upstream of the 5’ homologous region in the genome)/P5 (located within pZeoloxp) and P3 (located within pZeoloxp)/P5 (located downstream of the 3’ homologous region in the genome), were used to verify each gene. The successful amplification of both bands with the expected size indicated that the chromosomal integrations were correct. The amplification of one band with either primer pairs corresponded to single crossover recombination. The P3/P9 primer pair (Fig. 4A), located within the open reading frame, was used for further verification, and no band should be amplified for the correct disruptants.

Direct Disruption of OCH1 Gene

α-1,6-Mannosyltransferase, the product of OCH1 gene, adds the first α-1,6-mannose to the Man9GlCNac2 core oligosaccharide and initiates several subsequent high mannose-type N-glycosylation [18]. Accordingly, OCH1 is often chosen as the deletion target to avoid the hyperglycosylation of the expressed heterologous proteins in yeasts. However, OCH1 deletion is a very inefficient process according to previous studies. Choi and his colleagues [9] obtained only one P. pastoris strain with the inactivated OCH1 gene from approximately 1000 clones using double homologous strategy with 2878/1011 bp length of homologous flanks. Using homologous flanks as long as 3 kb, Vervecken et al. [15] failed to delete the OCH1 gene following the same strategy. To test the efficiency of OCH1 deletion, we first
used the direct one-step knockout method, and the results were used as a control group for the succeeding experiment.

We initially constructed a disruption vector for the OCH1 gene inactivation. This vector was generated by inserting two flanking regions, which were amplified from the P. pastoris GS115 genome, into the pZeoloxp module plasmid by performing a three-fragment ligation. The two homologous flanks, 5' and 3' regions of the disruption target, were 874 and 852 bp, respectively. The disruption plasmid, designated as pZeoloxp-OCH1, was then linearized at the NotI site and transformed into P. pastoris. More than 1000 clones were initially analyzed by PCR using the P1f/P1r and P2f/P2r primer pairs (Fig. 4A). Only one clone, designated as KO24#, showed one expected amplified fragment (1.42 kb). However, the other expected 1.25 kb fragment was not amplified from KO24#, suggesting that KO24# may be the result of a single crossover recombinant event at the 5’ homologous region.

**Construction of Plasmid pGKARSmazf-OCH1 for Modification of P. pastoris**

To apply our proposed strategy, we initially provided a backup OCH1 gene for P. pastoris before deletion to avoid compromising the fitness of the yeast cells because of function loss. This process was performed by cloning OCH1 into a carefully designed episomal pGKARSmazf-OCH1 vector (Fig. 3), which is characterized by three elements: 1) Pichia ARS2 (PARS2) fragment, which kept the plasmid inside the cells for a considerable time and allowed the easy removal when needed; 2) the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter; and 3) the mazf expression cassette regulated by promoter AOX1.
whose presence is essential for plasmid removal (as explained later).

The pGKARSmaf-OCH1 plasmid was introduced into the GS115 strain by electroporation without linearization. The transformants were screened on YPD plate that contains 500 mg L\textsuperscript{-1} of G418. Approximately 195 colonies were formed per μg of DNA. Colony PCR was performed using the two primer pairs, P\textsubscript{5}GAP/P\textsubscript{3}AOX1 and P\textsubscript{5}AOX1/P\textsubscript{3}AOX1, to verify the positive clones. Among the 16 clones, 15 showed the two expected bands, particularly 618 and 1837 bp for maf and OCH1 cassettes, respectively (data not shown).

![Figure 5. Comparison of cell growth of GS115 (shaded square), GS-tranOCH1-D\textsubscript{OCH1} (shaded circle), and GS-D\textsubscript{OCH1} (shaded triangle). Biological triplicate cultures of all three P. pastoris strains are grown at 30°C with 200 rpm shaking in 50 ml Falcon tubes containing 5 ml of YPD medium. Turbidity is monitored at OD\textsubscript{600}. doi:10.1371/journal.pone.0057952.g005](image)

### Table 2. Comparison of gene targeting efficiencies between conventional and new strategies.

| Recipient strain | Length of flankings (bp) (Left/Right) | Correct disruptants\textsuperscript{a} | Total clones | Frequency of targeting\textsuperscript{b} |
|------------------|---------------------------------------|----------------------------------------|--------------|------------------------------------------|
| **OCH1 gene deletion** |                                       |                                        |              |                                          |
| GS115            | 874/852                               | 0                                      | 1000         | 0%                                      |
| GS-tranOCH1      | 874/852                               | 6                                      | 60           | 10%                                     |
| **KU70 gene deletion** |                                       |                                        |              |                                          |
| GS115            | 911/976                               | 5                                      | 34           | 15%                                     |
| GS-tranKU70      | 911/976                               | 10                                     | 34           | 30%                                     |
| **SGS1 gene deletion** |                                       |                                        |              |                                          |
| GS115            | 1011/988                              | 1                                      | 103          | 1%                                      |
| GS-tranSGS1      | 1011/988                              | 8                                      | 34           | 24%                                     |

\textsuperscript{a}Correct disrupts are clones with correct gene targeting (i.e. gene replacement by homologous recombination) by PCR verification;

\textsuperscript{b}Frequency of targeting is defined as the ratio of number of correct disruptants to the number of total examined clones by PCR verification.

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Enhancement of the Gene Targeting Efficiency

Disruption of the Chromosomal OCH1 Gene and Elimination of the pGKARSmazf-OCH1 Plasmid

The transition host that carries the redundant OCH1 copies (designated as GS-tranOCH1) was used as the host for the OCH1 gene disruption. GS-tranOCH1 was transformed using the linearized pZeoloxp-OCH1 disruption plasmid based on the same procedure used in the direct one-step method. Approximately 60 transformants per µg of DNA were obtained, randomly picked, and analyzed by colony PCR using the P1u/P1r and P2u/P2r primer pairs. The results showed that the expected 1.42 kb band was amplified by the P1u/P1r primer pair from six clones, which were further analyzed using the P2u/P2r primer pair. All of the clones also revealed the expected 1.25 kb band (data not shown), suggesting that the chromosomal OCH1 was successfully deleted by gene replacement. The results were also confirmed by sequencing the 1.42 and 1.25 kb PCR products.

To remove pGKARSmazf-OCH1, och1 mutant transition strain (designated as GS-tranOCH1-Doch1) was streaked on a MM plate, in which the mazf could be expressed with the AOX1 promoter induced by methanol in the medium. The mazf gene, which was obtained from E. coli, encodes the MazF toxin that functions as an mRNA interferase and inhibits the growth of prokaryotes and eukaryotes [19,20]. Therefore, mazf expression likely causes a strong selection pressure on streaked strains and forces them to lose the obtained plasmids. The colonies, which were much smaller than that of the GS115 strain, appeared after the strain was cultured for 3 d at 30°C. A change in the colony morphology from smooth to rough appearance was also observed, which was consistent with the previous characterizations of och1 mutant strains [21]. Ten randomly selected colonies on the MM plate were determined by colony PCR with the P3u/P3r and P5u/P5r primer pairs to verify OCH1 deletion and plasmid clearance. All of the 10 clones exhibited the desired amplification pattern (Fig. 4B), in contrast to control (Fig. 4C), suggesting the efficient removal of helper pGKARSmazf-OCH1 plasmid from och1 mutant strains (resistant clones were named GS-Doch1).

The growth profiles of GS115, GS-tranOCH1-Doch1, and GS-Doch1 were then compared. The results showed that the duration of the lag phase of GS-Doch1 was approximately doubled compared with that of GS115 strain, indicating that the loss of the OCH1 gene is severely detrimental to yeast growth (Fig. 5). By contrast, the lag phase of GS-tranOCH1-Doch1 was slightly longer than that of GS115, suggesting that the och1 phenotype can be rescued by the presence of the redundant OCH1 gene in the episomal expression plasmid to a large extent.

Disruption of the Chromosomal SGS1 and KU70 Genes

To assess the effectiveness of our strategy, two P. pastoris genes, namely, SGS1 and KU70 were selected for gene-targeted deletion. The conventional one-step strategy and the new strategy were both applied. The disruption plasmids for SGS1 and KU70, namely, pZeoloxp-SGS1 and pZeoloxp-KU70, as well as two transition strains with the redundant copies of the corresponding genes, were generated based on the same procedure used in the OCH1 gene. Table 2 shows that the KU70 deletion by the one-step strategy resulted in five positive clones among the 34 selected transformants, whereas ten positive clones were obtained by performing the new strategy, indicating a 1-fold increase in the frequency of KU70 gene targeting. For SGS1, the frequency of obtaining positive clones was increased from 1% to 24% (Table 2), which is a 23-fold increase, compared with that in one-step strategy.

Discussion

For non-conventional yeasts, efficient gene targeting remains a challenge, which largely limits the study and the application of these industrially important strains. To address this issue, two strategies were most commonly employed: 1) increasing the HR efficiency, usually by increasing the homologous arm length. However, longer flanks are not always sufficient for a high percentage of homologous integration [22,23]; 2) suppressing the NHEJ pathway by deleting important functional proteins, such as KU70 and KU80, involved in the NHEJ pathway. For instance, the deletion of the KU70 gene in K. marxianus yields 80% homologous gene targeting efficiency by using homologous sequences with at least 40 bp in length [24]. The integration at HIS4 and ADE1 loci results in >90% targeting efficiencies with only 250 bp of flanking homologous DNA when the KU70 homolog of P. pastoris is knocked out [25]. Nevertheless, the stability and robustness of these strains should be further evaluated.

While previous studies have mainly focused on increasing the HR efficiency or decreasing the competition from the NHEJ pathway, one fact that is overlooked is that the strains after gene deletion on molecular and cellular basis, require a recovery and proliferation process to form colonies. Unfortunately, several non-conventional yeasts, such as P. pastoris, H. polymorpha, and K. lactis, are predominantly haploid. The deletion of functionally important genes, particularly when these genes do not have paralogs in the genome [26], often results in the loss of fitness, which inhibits the proliferation into sizeable colonies. This condition may be more evident under unfavorable conditions, such as cellular recovery from electroprotoplasm and growth on solid media with poor nutrition. For instance, the acs2 mutant of K. lactis requires three weeks to form large colonies [27]. Therefore, the possibility that the actual gene targeting efficiency can be reduced is high because of the unfurmed colonies.

Based on this rationale, we aimed to improve the fitness of haploid yeast cells by providing backup genes presented in a well-designed episomal vector. The fitness of mutant cells was improved effectively by this strategy. The growth defect of och1 mutant cells was restored to a large extent. As a result, the improvement of gene targeting efficiencies with this strategy is significant. The och1 disruptants, which cannot otherwise be acquired in practice by direct one-step deletion [15], can be obtained at a frequency of approximately 10% with mediate length of homologous flanks. The efficiencies of the other two genes, KU70 and SGS1, were also increased by 1- and 23-fold, respectively. These results validated our assumption that cellular fitness is an important factor that limits the efficiency of gene targeting in non-conventional yeasts.

In summary, we provided an efficient gene targeting strategy for non-conventional yeasts. The targeted gene was initially amplified by cloning into a helper expression plasmid. After the gene was successfully deleted from the genome, the helper plasmid was removed. Thus, the och1 disruptants were obtained at a frequency of 10%. The gene targeting efficiencies of SGS1 and KU70 were increased by 1- and 23-fold, respectively.

Supporting Information

Table S1 Primers used in this study.

(DOC)
Author Contributions
Conceived and designed the experiments: CZ TZ. Performed the experiments: CZ HS PL. Analyzed the data: CZ TZ YL. Wrote the paper: CZ TZ NH YL.

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