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Disruption of *URA3* Gene in Thermotolerant Natural Yeast by CRISPR/Cas9 Technology

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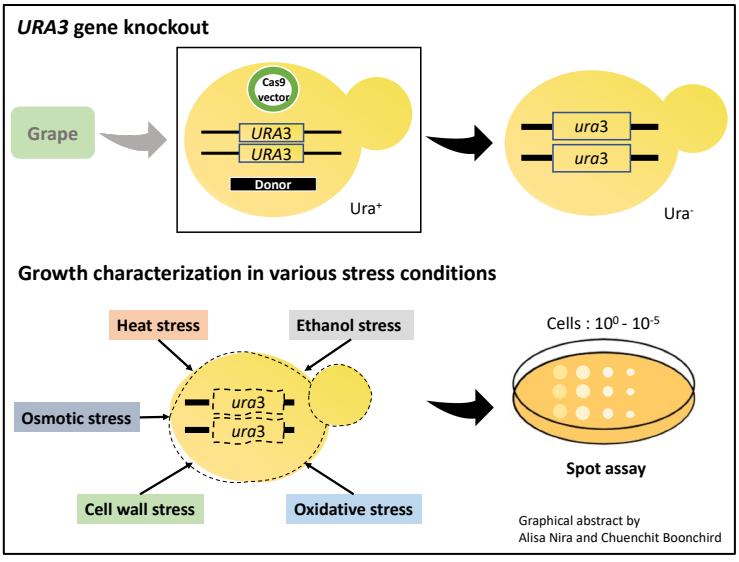
Abstract: Thermotolerant yeasts from natural sources have received much attention in industry as they are robust and can tolerate several stresses. To apply natural yeast strain as a host for production of heterologous protein, genetic marker is required for the selection of transformed cells and maintenance of recombinant plasmids. The yeast *Saccharomyces cerevisiae* strain C3253 isolated from grape in Thailand was previously characterized as thermotolerant yeast which can tolerated multiple stresses. This study aimed to construct a *ura*3 auxotrophic mutant via CRISPR/Cas9 technique which then could be transformed by yeast plasmid with a *URA*3 selectable marker. *URA*3 gene of this yeast strain was knocked out by inserting a plasmid containing a Cas9 gene under control of *GAP*1 promoter, a guide RNA expression cassette controlled by *SNR*52 promoter for specific target sites of CRISPR/Cas9, *Kan*Mx4 selectable marker, and double-stranded oligonucleotide repair template (Donor). From the observation of uracil required colonies in various selective medium as well as confirmation by DNA sequencing at the target region, the results demonstrated that *URA*3 gene in the natural yeast strain was completely knocked out. The mutant clones retained characteristic of multiple stress tolerance, i.e., heat, oxidative, osmotic, ethanol and cell wall stresses. This study suggests that CRISPR/Cas9 technique works efficiently to knockout gene of natural yeast strain for further applications.

Graphical abstract:



Keywords: Thermotolerant; *Saccharomyces cerevisiae*; CRISPR-Cas9; *URA*3; 5-FOA

1. Introduction

Yeasts have been broadly used in food production such as in bakery and alcoholic beverages, and in healthcare sectors to produce biopharmaceutical proteins as they are generally recognized as safe (GRAS) microorganisms [1]. For the production of recombinant proteins, the conventional yeast *Saccharomyces cerevisiae* has been used since it is easy to genetically manipulate, grows in simple media, and has an ability of post-translational modification of proteins playing a key role in functional proteomic [2, 3]. Consequently, *S. cerevisiae* strain has been extensively modified via genetic engineering to improve the strains for all industrial applications [4]. However, current recombinant strains used in industry have limitations including the instability of the desirable phenotype [5]. Therefore, finding new yeast strains in nature is interesting as they may be more efficient in higher stress tolerance and well growth without having to be modified compared to the original strains. In large scale production, yeasts encounter the problem of heat generated during fermentation process, which needs the cooling of fermenters for constant fermentation with microbes [6, 7]. Therefore, thermotolerant yeasts are expected to be employed at high temperature fermentation for several applications to reduce risk of contamination, as well as cooling and operating cost [8-10]. Typically, the optimum growth temperature of yeast is 30 °C whereas Thermotolerant yeast grows at 35–45 °C which is 5–10 °C higher than mesophilic strains [6].

To introduce new features into the yeast cells, normally auxotrophic mutant is used as a host strain [11, 12]. Nevertheless, novel yeast strains isolated from natural sources carry no auxotrophic mutant for further introducing heterologous genes. There are several approaches to delete target genes including PCR – based gene disruption, λ Red Recombination Strategy [13], Homologous Recombination, Zinc Finger Nucleases, Transcription Activator-like Effector, and Clustered Regularly Interspaced Palindromic Repeats (CRISPR) [14-16]. Among these techniques, CRISPR/Cas9 is the most powerful gene editing tools for fundamental and preclinical research in terms of simplicity and rapidity [17].

The components of CRISPR/Cas9 type II system are Cas9 protein bonded and guided to a target sequence based on a complementary base-pairing rule by a single guide RNA (sgRNA or gRNA), a fusion of a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA), which is modified to have proper sequences. The ribonucleoprotein complex between Cas9 and sgRNA generates a double-strand break (DSB) at specific region of DNA adjacent to a protospacer-adjacent motif (PAM), NGG sequence, and then the recognition site can be repaired by non-homologous end joining (NHEJ) or a homology directed recombination (HDR) mechanism [18]. However, the CRISPR/Cas9 system can cause off-target effects generating undesirable multiple DSBs [19], but nowadays there are several computational tools to select and validate sgRNAs, predict off-target loci, analyze editing outcomes, and others [20-23].

This study aims to generate an auxotrophic mutant, *ura*3, in the thermotolerant natural isolate yeast via CRISPR/Cas9 type II system. Guide RNA expression vector consisted of yeast vector backbone as well as guide RNA expression cassette designed via web tools was constructed. Then, the double-stranded oligonucleotide repair template (Donor) was designed correlating with target region and gRNA target sequence to be used in yeast transformation process for knocking out *URA*3 gene via homologous recombination (HR). In addition, the growth characteristics of *ura3* knockout strain was confirmed under various stresses including heat, oxidative, osmotic, ethanol, and cell wall stresses by spot dilution assay.

2. Materials and Methods

2.1 Strains, media and plasmid

Escherichia coli DH5 alpha was applied in the process of vector construction. *S. cerevisiae* thermotolerant strain C3253-1D used in this study was homozygous diploid strain which

derived from tetrad analysis of the original strain C3253 provided by the yeast collection of the Department of Biotechnology, Faculty of Science, Mahidol University. Laboratory strain W303 and thermotolerant strain C3723 were employed as controls [22]. LB medium supplement with ampicillin 100 μg/mL was used for *E. coli* cultivation. The media for yeasts were yeast peptone dextrose medium (YPD) consisting of yeast extract (1%), peptone (2%), dextrose (2%), agar (2% if required); synthetic dextrose medium (SD) composing of yeast nitrogen based without amino acids (0.67%), dextrose (2%), agar (2% if required) supplemented with uracil (20 mg/L), and 5-Fluoroorotic Acid (5-FOA; 0.1%) as appropriated [24]. A plasmid pML104-*Kan*Mx4 was purchased from Addgene (http://n2t.net/addgene:83476; RRID:Addgene_83476).

2.2 Construction of guide RNA expression plasmid (pgRNA-URA3)

The protocol of the construction of guide RNA expression vectors using pML104-KanMx4 vector carrying Cas9 gene was described by Laughery *et al*., 2015 [25]. *URA*3 DNA sequence was retrieved from website yeastgenome.org. Oligonucleotide 1 and 2 for construction of guide RNA expression cassette were designed via bioinformatic tools including CRISPR-Cas9 primer design Website (http://wyrickbioinfo2.smb.wsu.edu/crispr.html), CHOPCHOP web tool, and CRISPRdirect-Rational design of CRISPR/Cas target. The sequences of the guide RNA expression cassette in this study were illustrated in Table 1. Both oligonucletides were hybridized and inserted into pML104-*Kan*Mx4 at *Bcl*I and *Swa*I sites to obtain pgRNA-URA3 plasmid. The constructed plasmid was transformed into *E. coli* DH5*α* described by NEB: New England Biolabs with minor modification. The transformants were confirmed by *E. coli* colony PCR and/or DNA sequencing. The overall process was shown in Figure 1. The constructed plasmid was extracted by minipreparation [25]

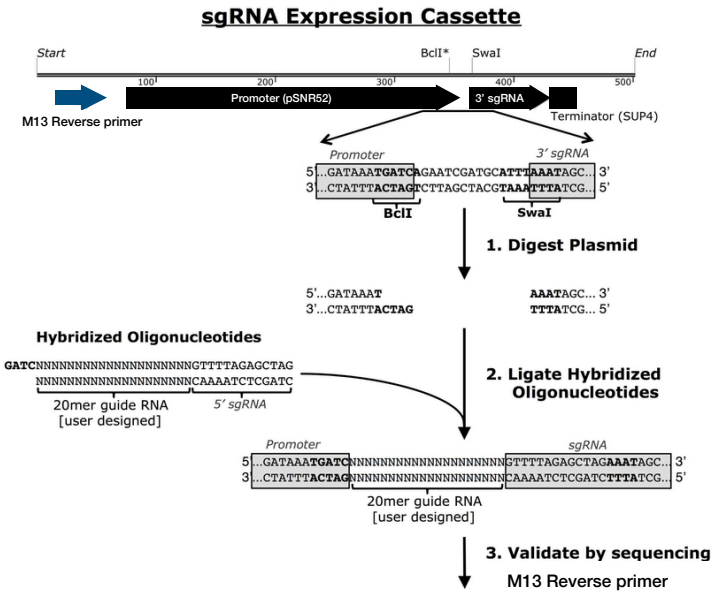


Figure 1. Protocol for cloning user-designed guide sequence into sgRNA expression cassette in pML104-*Kan*Mx4 vector. The figure was modified from Laughery et al. [25].

2.3 E. coli colony PCR and DNA sequencing of guide RNA in pgRNA-URA3 plasmid

The colony PCR was performed in reaction mixture consisting of 1x reaction buffer, 1.5-3 mM MgCl2, 200 μM dNTP Mix, 1-2.5 U Taq DNA Polymerase, 0.2-1 μM T3 promoter forward and M13 reverse primers (shown in Table1), 0.2-1 μM oligonucleotide 2, nuclease free water, and one colony approximately 1 mm in diameter as source of DNA template.

The cycling program composed of one cycle of 95 °C for 5 min, 25-35 cycles of 95 °C for 30 sec, 52 °C for 30 sec, 72 °C for 30 sec, and then incubation at 72 °C for 5 min, and a last incubation at 4 °C. To verify DNA sequence of guide RNA, the plasmid was sequenced using M13 reverse primer and nucleotide sequence was analyzed by sequence alignment tools.

2.4 Construction of double-stranded oligonucleotide repair template

Generally, long oligonucleotides, not less than 90 nts having at least 30-40 nts of homologous flanking DNA on both sides, are used as repair templates in yeast genome editing. The oligonucleotides should contain target sequences (a specific part of *URA*3 gene) having some mutations of the PAM motif (5′ NGG 3′) and/or 20-bp target sequence of sgRNA to prevent recutting of Cas9 protein. It contained mutations with six bases change associated with disrupting the PAM motif and crRNA targeting sequence and stop codon shown in Figure 3.

2.5 Transformation of S. cerevisiae for CRISPR–Cas9 gene editing

Yeast cells were transformed by PEG/LiAc method by the protocol of Assoc. Prof. M. Sugiyama, Osaka University (personnel communication). Briefly, fresh yeast cells were cultivated in YPD broth at 30 °C with shaking at 200 rpm for 16 h. Then, culture was transferred into fresh YPD broth and grown at the same condition for 4 h (to reach mid-log phase; OD_{600} = ca.1.0). Cells were harvested by centrifugation at 2,000 rpm for 3 min. After washing the cell pellets with sterile water and 0.1 M LiAc, respectively, cells were incubated in 0.1 M LiAc at 30 °C for 10 min. Subsequently, 0.1 mL of cells were mixed with 0.24 mL of 50% (w/v) polyethylene glycol 4,000 (PEG), 0.036 mL of 1.0 M LiAc, 5 μL of carrier DNA (10 mg/mL), and 2.5 μg of guide RNA expression vector, 10 ng/μL of the donor, together with sterile water, respectively. The cell mixture was incubated at 30 °C for 30 min. Then, the tube was heat shock at 42 °C for 20-25 min. The cell pellets were collected and further incubated in 1 mL of YPD broth at 30 °C for 16 h without shaking. Finally, the cells were harvested, resuspended with 0.1 mL of water and spread onto YPD medium containing 250 μg/mL G418 (YPD + G418). Transformants were grown at 30 °C for 2-3 days to select $G418^r$ colonies.

Table 1. List of primers used for *URA*3 gene knockout

Name	Sequences (5′ > 3′)	Length (bp), Tm (°C)	Purposes
gRNA_URA3 Fw (Oligonucleotide 1)	GATCTGTAGAGACCACATCATCCAGTTTTAGAGCTAG	37, 62	To construct a guide RNA expression cassette
gRNA_URA3 Rw (Oligonucleotide 2)	CTAGCTCTAAAACTGGATGATGTGGTCTCTACA	33, 60.7	
T3 promotor	GCAATTAACCCTCACTAAAGG	21, 53	To verify guide RNA expression cassette
M13 reverse	AGCGGATAACAATTTCACACAGG	23, 58	To verify guide RNA expression cassette
URA3_down_FW	ATAACTAATTACATGATGTCGAAAGCTACATATAA	35, 66	To verify *ura*3 knockout by sequencing
URA3_RW	TTAGTTTTGCTGGCCGCATCTTCT	24, 63.5	

2.6 Validation of ura3 knockout

Random $G418^r$ clones were re-streaked onto YPD + G418 medium and incubated at 30 °C for 24 h. Then, $G418^r$ clones were confirmed for the deletion of *URA*3 gene by growth

ability on SD, and SD plus uracil media. The guide RNA expression vector was cured by counter-selection on SD plus uracil and 5-FOA medium. Curing of plasmid was confirmed as $G418^s$ on YPD + G418 medium. The target gene was verified by DNA sequencing (Macrogen Inc., Korea) using URA3_down_FW and URA3_RW primers (shown in Table1). The DNA sequence was analyzed by sequence alignment tools, Clustal Omega.

2.7 Spot dilution assay

Firstly, a yeast colony was grown in YPD broth at 30 °C with shaking for 16 h. The cell density was measured at OD_{660} and diluted to obtain the initial OD_{660} equal to 1.0. Then, a 10-fold serial dilution in sterile distilled water was made till 10^{-5} dilution. A 4 µL of diluted culture were dropped on YPD agar plates containing 12 mM H_2O_2, 30% dextrose, 17% ethanol, or 25 mg/L calcofluor white (CFW), and then incubated at 30 °C for 1 days, except the plates having 17% ethanol were incubated at 30 °C for 2 days. To examine the growth at high temperature, the plates were incubated at 39 °C.

3. Results

3.1. Cas9 genome editing in the thermotolerant yeast

3.1.1. Guide RNA expression plasmid construction

The insertion of gRNA expression cassette into pML104-KanMx4 vector at *Bcl*I and *Swa*I sites was successful which confirmed by *E. coli* colony PCR, and sequencing of plasmid extracted from *E. coli* transformants. The constructed plasmid harboring gRNA expression cassette to knock down *URA*3 gene was illustrated in Figure 2.

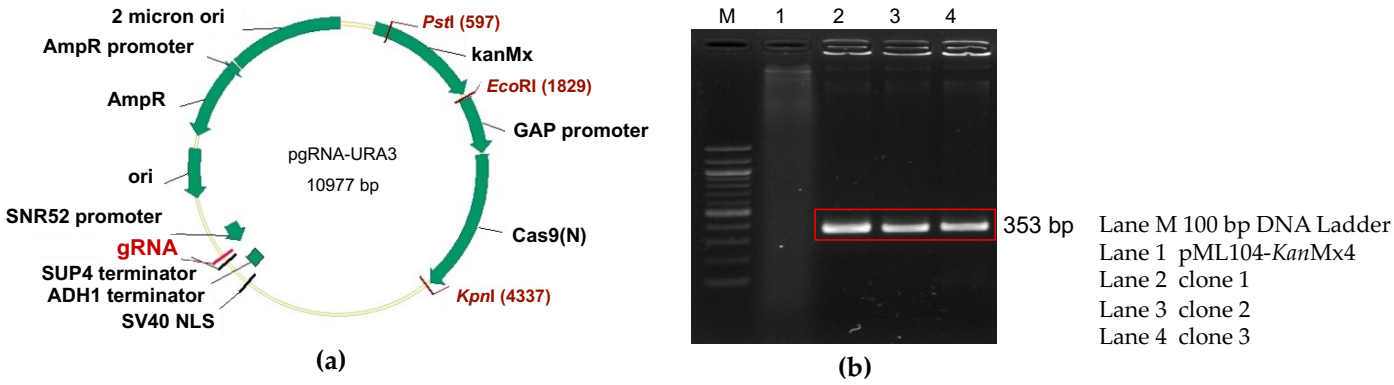


Figure 2. (**a**) Plasmid map of guide RNA expression plasmid (pgRNA-URA3) (modified from Addgene); (**b**) Verification of gRNA expression cassette by *E. coli* colony PCR

3.1.2. Double-stranded oligonucleotide repair template construction

In this study, the oligonucleotides repair template correlated with the guide RNA expression cassette contained six bases change, which were not only associated with disrupting the PAM motif and crRNA targeting sequence, but also inducing stop codon, as shown in Figure 3.

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5′
GCATCCCTTCCCTTTGCAAATAGTCCTCTTCCAACAATAATAATGTCAGATCCAGTAGATACCACGTCATCCTATGT
TCTATACTGTTGACCCAATGCGTCTCCCTTGTCATCTAAACCCACACC 3′

                                                    Stop codon
3′
CGTAGGGAAGGGAAACGTTTATCAGGAGAAGGTTGTTATTATTACAGTCTAGGTCATCTATGGTGCAGTAGGATACA
AGATATGACAACTGGGTTACGCAGAGGGAACAGTAGATTTGGGTGTGG 5′
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Figure 3. The double-stranded oligonucleotide repair template (125 bp) for gRNA expression cassette in this study

3.1.3. Construction of *ura*3 knockout homozygous diploid strain

After transformation of the guide RNA expression plasmid and double-stranded oligonucleotide repair template into yeast strain C3253-1D, 64 G418^r clones were selected. The result showed that almost all of the G418^r clones (63 clones) grew well in the presence of G418 which confirmed that the guide RNA expression plasmid was effectively expressed. By counter selection on 5-FOA medium, around 50 clones grew well on SD plus uracil with and without 5-FOA media but were not able to grow on SD medium as shown in Figure 4. This result implied that *URA*3 gene was completely knocked out. Moreover, by observation of growth on YPD plus G418, all colonies were sentitive to G418 which demonstrated that the guide RNA expression plasmid was cured in the process of counter-selection. Growth of representative *ura*3 knockout clones was displayed in Figure 4. Three *ura*3 knockout clones were randomly selected to confirm the *URA*3 target by DNA sequencing. Finally, auxotrophic mutant at *ura*3 gene of the thermotolearant yeasts was successful created by CRISPR/Cas9.

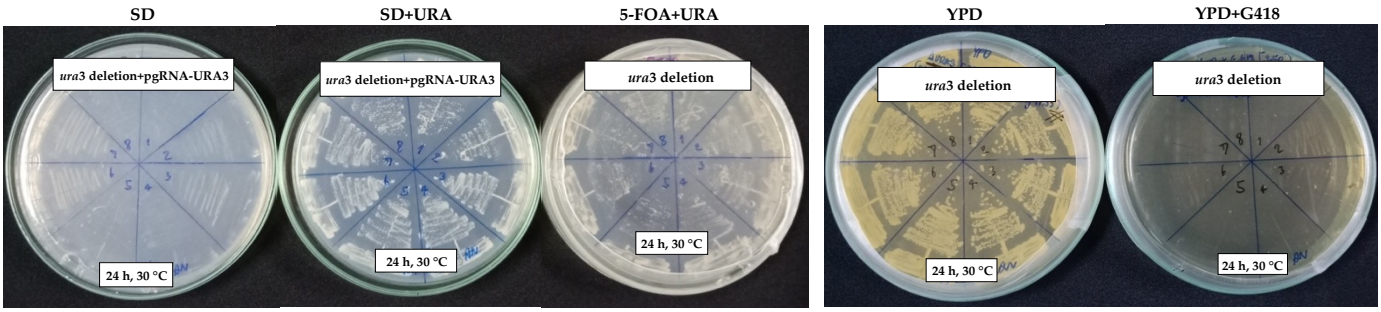

Figure 4. Verification of *ura*3 knockout clones grown on different medium for (a) loss of *URA*3 function and (**b**) curing of pgRNA-URA3 plasmid harboring *Kan*Mx4 marker.

3.2. Growth characteristics of ura3 knockout thermotolerant yeast in various stress conditions

The results in Figure 5 illustrated that *ura*3 knockout strain (Δ*ura*3 C.1 - Δ*ura*3 C.3) could grew in multiple stresses similar to the original strain (C3253-1D) and the positive control C3723. The laboratory strain W303 was not able to grow in all stress conditions.

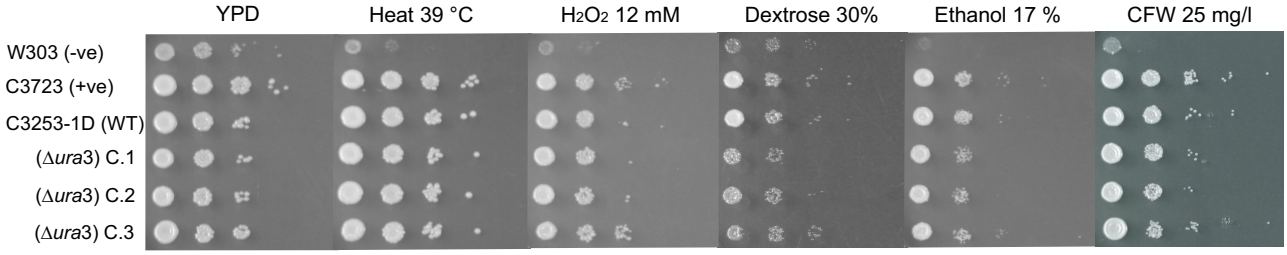

Figure 5. Growth of *ura*3 knockout *S. cerevisiae* strains in various stress condition.

4. Discussion

Knockout of *URA*3 gene was chosen for creating auxotrophic mutant in thermotolerant yeast because the selection of *ura*3 was simply performed by counter selection on medium with 5-FOA (www.yeastgenome.org).

This study demonstrated that targeting knockout of *URA*3 gene in the homozygous diploid strain (C3253-1D) was effectively created using the guide RNA expression plasmid (pgRNA-URA3) together with the donor sequences. Growing transformed cells on YPD medium containing glucose as C source allowed the expression of Cas9 gene by

*ADH*1 promoter and guide RNA by *SNR*52 promoter (www.yeastgenome.org). The number of positive transformants, after re-streaked onto selective medium, was high up to 98 percent. This event also observed in the report by Laughery et al. [25] testing pML104 and pML107 vectors efficacy for CRISPR–Cas9 genome editing to knockout *TRP*1 locus. After using both TRP1–sgRNA expression vector with double-stranded oligonucleotide template, the yield of edited transformants (*trp*1) was high up to 97%. Previous research reported that off-target effects can occur because of the toxicity of CRISPR-Cas9 activity which resulted in decreasing the transformation efficiency [26]. However, our result encountered low off-target effects which around 2% of unedited transformants was obtained. The application of counter selection using 5-FOA has been widely used for selection of yeast mutant from wild type because the chemical was toxic to wild type which *URA*3 gene still functions.

In large-scale production such as bioethanol fermentation, yeast has to face a variety of stresses in terms of fluctuations in osmolarity, ethanol concentration, temperature, and others [27]. Therefore, the growth characteristics was also evaluated in this study after obtaining the *ura*3 knockout strain. The results showed that all *ura*3 knockout clones could tolerated multiple stresses compared with the laboratory strain W303 which was sensitive to all stresses. To confirm that C3253-1D as well as *ura*3 knockout were thermotolerant strains, the control strain C3723 was compared. The results from spot dilution assay clearly showed that *ura*3 knockout clones could tolerate all stress conditions, especially heat stress, 39 °C. In addition, the results of the growth characteristics of every clone correlated to previous studies [28].

5. Conclusions

The thermotolerant natural isolate yeast C3253-1D was completely disrupted at 2 copies of *URA*3 gene via CRISPR/Cas9 technique. The result suggests that C3253-1D *ura*3 is suitable to apply as host strain for introducing recombinant plasmid for heterologous protein production. Moreover, this strain has potential for large-scale productions due to the property of stress tolerance.

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Conflicts of Interest: The authors declare no conflict of interest.

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