

Generation of a Uracil Auxotroph Strain of the Probiotic Yeast *Saccharomyces boulardii* as a Host for the Recombinant Protein Production

Hassan Hamed ¹, Ali Misaghi ¹, Mohammad Hossein Modarressi ², Taghi Zahraei Salehi ³, Dorsa Khorasanizadeh ⁴, and Vahid Khalaj ^{4*}

1- Department of Food Hygiene, Faculty of Veterinary of Medicine, University of Tehran, Tehran, Iran

2- Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran

3- Department of Microbiology, Faculty of Veterinary of Medicine, University of Tehran, Tehran, Iran

4- Fungal Biotechnology Group, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

Abstract

Background: *Saccharomyces boulardii* (*S. boulardii*) is the best known probiotic yeast. The genetic engineering of this probiotic strain requires the availability of appropriate mutants to accept various gene constructs carrying different selection markers. As the auxotrophy selection markers are under focus, we have generated a *ura3* auxotroph mutant of *S. boulardii* for use in further genetic manipulations.

Methods: Classical UV mutagenesis was used for the generation of auxotroph mutants. The mutants were selected in the presence of 5-FOA (5-Fluoroorotic acid), uracil and uridine. Uracil auxotrophy phenotype was confirmed by the ability of mutants to grow in the presence of uracil and the lack of growth in the absence of this compound. To test whether the uracil auxotrophy phenotype is due to the inactivation of *URA3*, the mutants were transformed with a plasmid carrying the gene. An *in vitro* assay was used for the analysis of acid and bile resistance capacity of these mutants.

Results: Three mutants were found to be *ura3* auxotroph as they were able to grow only in the presence of uracil. When the *URA3* gene was added, these mutants were able to grow normally in the absence of uracil. Further *in vitro* analysis showed that the acid and bile resistance capacity of one of these mutants is intact and similar to the wild type.

Conclusion: A uracil auxotroph mutant of the probiotic yeast, *S. boulardii*, was generated and characterized. This auxotroph strain may have potential applications in the production and delivery of the recombinant pharmaceuticals into the intestinal lumen.

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Introduction

As the knowledge of probiotics expands, more possibilities arise for the engineering of the new probiotic strains. Recombinant probiotics are being considered as efficient bio-systems for the delivery of active molecules to the intestinal mucosa ¹. *S. boulardii* is a well known probiotic yeast which is used

alone or in combination with probiotic bacteria to support digestive system ²⁻⁶. *S. boulardii* is often marketed in a lyophilized form and is called *S. boulardii* *lyo*. The availability of well-established genetic engineering methods in yeast has facilitated the possible genetic manipulation of this probiotic yeast.

In genetic manipulation procedures, the selection of recombinant strains is usually performed by employing a suitable selection marker on a plasmid carrying the gene construct. Antibiotic resistance markers are widely used, but are considered as a major concern in probiotic applications. Hence, it is necessary to remove the antibiotic resistant gene from the host prior to commercial application⁷. In this sense, the auxotrophic markers may be a better substitute as they are indigenous⁸. Although these selection markers are commonly used in practice, but they require appropriate host strains which are auxotrophic for the specific nutrients corresponding to the inactivated gene⁹. One example of these markers is the *URA3* gene that encodes orotidine 5-monophosphate decarboxylase (OMPD Case), an enzyme involved in the *de novo* synthesis of pyrimidine ribonucleotides¹⁰. The inactivation of *URA3* results in uracil auxotrophy and 5-fluoroorotic acid resistance phenotype¹¹.

In the present study, a uracil auxotroph mutant of *S. boulardii* was generated through UV mutagenesis. The auxotroph mutant was complemented by the *URA3* gene. The *ura3⁻* mutant strain of *S. boulardii* can be used in future engineering of this important probiotic yeast.

Materials and Methods

Strains, media and plasmids

The yeast and bacterial strains used in the present study are listed in table 1. pGEM-T Easy cloning system (Promega) was used for the cloning of PCR products. Plasmid pYES2

(Invitrogen) containing *Saccharomyces cerevisiae* (*S. cerevisiae*) *URA3* gene was used as a control in transformation experiments.

Yeasts strains were grown and kept in YPD medium (1% yeast extract, 2% polypeptone and 2% dextrose). Yeast Nitrogen Base with ammonium sulphate and without amino acids (YNB medium; Sigma-Aldrich) was prepared at a concentration of 0.67% and was supplemented with 2% glucose, 10 mM uridine and uracil, and 0.1% 5-FOA (Sigma) to use in screening of auxotrophs.

DNA manipulations

Genomic DNA from both *S. cerevisiae* $\Sigma 1278b$ and *S. boulardii* was prepared as described before¹². All PCRs were performed as 30 cycles of 95°C for 1 min, 58°C for 30 s and 72°C for 1 min. The *S. cerevisiae* actin fragment (500 bp) was amplified using primers ACT1_F (CCCAATTGAACACGGTATTG) and ACT1_R (GCAGCGGTTTGCATTCTTG) as a control in PCR reactions (Table 2).

UV light mutagenesis and isolation of uracil auxotrophs

A single colony from *S. boulardii* parental strain was grown for 20 hr in YPD broth. Cells were collected and washed with PBS and subjected to UV mutagenesis. 20 ml of cell suspension (1×10^7 viable yeasts ml^{-1} in PBS) was gently agitated by a magnetic flea in a glass petri dish (with the lid removed) 15 cm below a UV lamp (Philips, TUV 15W/G15). A dose response experiment was carried out by removing 0.5 ml samples at 10 s intervals over a 100 s period. Irradiated cell suspensions were stored in foil-wrapped tubes at 4 °C overnight to avoid photoreactivation.

Table 1. Strains used in this study

Strain of <i>S. cerevisiae</i>	Genotype	Source
<i>S. cerevisiae</i> $\Sigma 1278b$	Wild-type (Mata)	Our laboratory
<i>S. boulardii</i>	Wild type (subspecies <i>lyo</i>)	DiarSafe, (Wren Laboratories Ltd)
<i>S. boulardii</i> M1	<i>ura3⁻</i>	This study
<i>S. boulardii</i> M2	<i>ura3⁻</i>	This study
<i>S. boulardii</i> M3	<i>ura3⁻</i>	This study
Strain of <i>E. coli</i>		
<i>E. coli</i> Top10	F' { <i>lacIq</i> Tn10 (TetR)} <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) $\Phi 80$ <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Invitrogen

Table 2. Primers used in this study

Primer name	Sequence
ACT1_F (sense)	5' CCCAATTGAACACGGTATTG3'
ACT1_R (antisense)	5' GCAGCGGTTTGCATTTCTTG3'
URA3_F (sense)	5' GTTAATGTGGCTGTGGTTTC 3'
URA3_R (antisense)	5' GTTACTTGGTTCTGGCGAGG3'

Dilutions of cell suspension from various exposure times were made and plated onto YPD agar (3 replicates per dilution). All plates were incubated in the dark at 30 °C. Colonies were counted initially after two days and finally after four days of incubation. A kill curve was plotted to estimate the exposure time to UV light to kill 90% of cells. This was then used for the subsequent mutagenesis procedures and the UV irradiated cells were kept at 4 °C in a foil-wrapped tube.

To isolate *ura3* auxotroph mutants, approximately 10^7 mutagenized cells were spread onto 5-FOA plates containing uracil and uridine, and then were incubated at 30°C up to one week. The recovered colonies were isolated and plated on YNB medium with or without uracil supplement. Uracil auxotroph mutants were detected by their ability to grow only in the presence of this chemical.

Construction of URA3 cassette

The *S. cerevisiae* *URA3* sequence (URA3/YEL021W, yeast genome database) was used as a template to design the *URA3* specific primers. The forward primer, *URA3_F* (5'-GTTAATGTGGCTGTGGTT TC-3'), and the reverse primer, *URA3_R* (5'-GTTACTTGG TTC TGGCGAGG-3') (Table 2), were designed to amplify an approximately 1.2 kb *URA3* fragment containing the entire coding sequence with 5' and 3' flanking regions.

PCR on genomic DNA of *S. boulardii* was carried out using these primers. The resulting PCR fragment was cloned into pGEM-Teasy vector. The final vector was called pGEM-ura3 and used in transformation of the auxotroph strains.

Transformation of *S. boulardii* auxotroph strains

S. boulardii auxotrophic yeasts (*ura3*⁻) were transformed with the plasmid pGEM-ura3 us-

ing a standard electroporation method¹³. As a positive control, a commercial episomal vector, pYES2 (Invitrogen), was used in transformation experiments. Following the transformation, cells were plated on YNB agar medium lacking uracil and uridine supplements.

Bile and acid resistance assay

Resistance tests were performed as described by van der Aa Kühle¹⁴. In brief, all strains were refreshed in MYGP medium (Malt extract 1%, Yeast extract 1%, Peptone 2%, Dextrose 2%) for 24 hr at 30 °C. Assays were performed in a 200 µl volume in 96-microwell plates. The wells were inoculated in triplicates with 10^6 yeast cells and the cells were allowed to grow for 48 hr at 30 °C in YNB medium containing acid (pH=2.5) or 0.3% (w/v) Oxgall (Difco). For the auxotroph mutants the medium was supplemented with uracil (10 mM). Viability tests were performed after 4 hr of incubation by plating of 100 µl of cell suspensions onto MYGP agar for 3 days at 30 °C.

Results

UV survival curve

UV irradiation of cell suspension from *S. boulardii* was performed and the percentage of survival against time was plotted to estimate the UV exposure time required to kill 90% of cells (Figure 1). An exposure time of 23 s was chosen for mutagenesis.

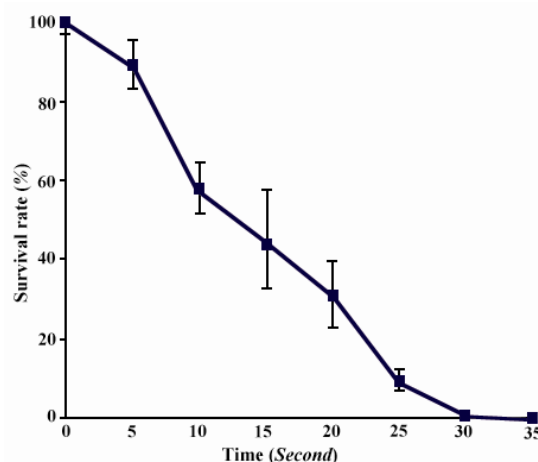


Figure 1. Survival rate of *S. boulardii* following UV irradiation. Cell counts were performed in triplicates

Isolation of *URA3* mutants

Mutagenized yeasts were screened for *ura3⁻* phenotype on 5-FOA/UU plates. Approximately 350 FOA resistant colonies were isolated and further screened for uracil auxotrophy (Figure 2). Eight colonies out of 350 were able to grow in YNB-uracil medium but not in YNB. Three mutants, *S. boulardii* M1, M2 and M3, which had similar growth properties compared to the wild type, were chosen for further studies. These mutants were tested for mutation reversion by plating of 10^6 , 10^7 and 10^8 viable cells on YNB agar and counting the number of possible revertants up to 5 days. No revertant was appeared during these incubation periods, indicating that a stable mutation has occurred in the target gene.

Complementation of *ura3⁻* mutants of *S. boulardii*

The *URA3* gene from *S. boulardii* was successfully amplified as a 1.2 kb fragment using designed specific primers (Figure 3). This was subsequently cloned into pGEM-Teasy vector. The final construct, pGEM-ura3, was confirmed by restriction analysis and sequencing. The size of *URA3* construct was 4.3 kb and the digestion map using *SacI*/*NcoI* showed two expected fragments as ~1 kb and 3.3 kb (Figure 4B).

The potential *ura3⁻* mutants of *S. boulardii* (M1, M2 and M3) were transformed with pGEM-ura3 vector. As a positive control, the same mutants were transformed with an episomal vector, pYES2, containing *URA3* as a selectable marker (Figure 5A). Both trans-

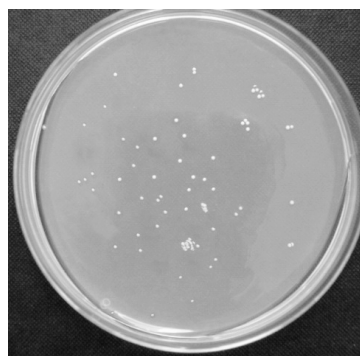


Figure 2. FOA resistant colonies on YNB plates. Approximately 10^7 mutagenized cells (10% survival) were plated on YNB-FOA -UU plates. The resistant colonies appeared after 5-7 days

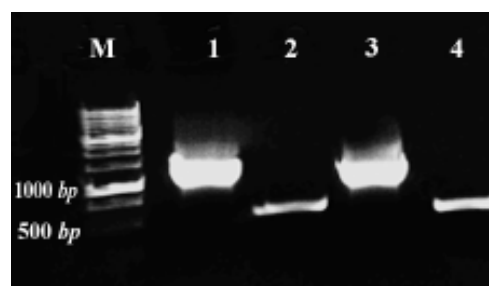


Figure 3. Amplification of *ura3* and actin fragments using genomic DNA of *S. cerevisiae* and *S. boulardii*. M: Size marker, 1: *S. cerevisiae* *ura3* fragment (1.2 kb), 2: *S. cerevisiae* actin fragment (0.5 kb), 3: *S. boulardii* *ura3* and 4: *S. boulardii* actin fragments

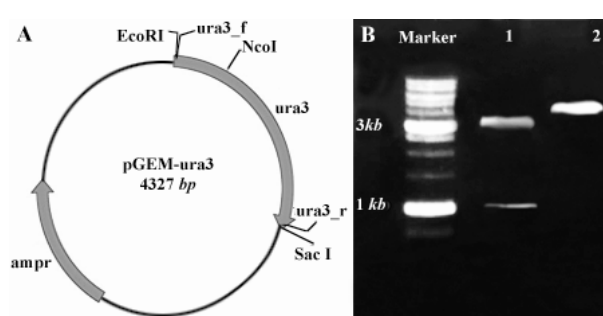


Figure 4. A) Schematic representation of pGEM-ura3 construct. The position of designed primers and restriction sites is shown. B) Restriction analysis of pGEM-ura3 vector. Lane 1: Fragments generated by *NcoI*/*SacI* digestion of the construct (~1 kb and ~3.3 kb). Lane 2: *NcoI* linearized plasmid

formations were efficient and resulted in several hundred transformants from each single reaction (1 μ g of each plasmid per reaction).

To confirm that the pYES2 construct is present in these *ura3⁺* transformants, plasmid DNA was extracted from one of these transformants and subjected to restriction analysis using *EcoRI* and *ClaI* Enzymes. The results confirmed that the isolated plasmid is intact and identical to original plasmid, pYES2 (Figure 5B).

Acid and bile resistance in auxotroph mutants

The ability of auxotroph mutants to resist pH=2.5 and 0.3% oxgall was assessed. Table 3 shows the growth phenotype of three different mutants compared to the wild types. All tested strains showed different resistance against acid and bile. Among the mutants, only *S. boulardii* M2 showed a resistance pattern similar to the *S. boulardii* wild type.

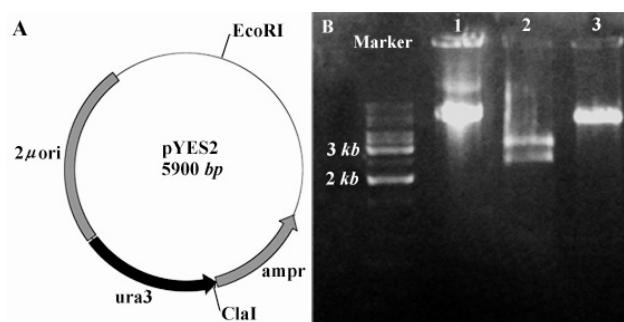


Figure 5. A) Schematic representation of pYES2 vector. pYES2 contains the auxotrophic marker *URA3*, 2 μ origin and the ampicillin resistance marker. B) Restriction analysis of pYES2. Lane 1: undigested plasmid. Lane2: *EcoRI/ClaI* digested vector showed two expected bands of ~2.5 and ~3.2 kb. Lane 3: *ClaI* linearized plasmid (5.9 kb)

Table 3. Growth ability of wild type strains and *ura3* mutants in the presence of acid and bile

Yeast strain	Growth ^a	
	pH=2.5 ^c	0.3% Oxgall
<i>S. boulardii</i> wild type	+	++
<i>S. boulardii</i> M1 ^b	+	+
<i>S. boulardii</i> M2	+	++
<i>S. boulardii</i> M3	—	+
<i>S. cerevisiae</i> Σ 1278b	—	+

A) -no growth; + growth delay>4 hr; ++no delay in growth.

B) M1, M2 and M3:the *ura3* mutant of *S. boulardii*

C) Survival after 4-hr incubation at pH=2.5

Discussion

The design, creation and genetic manipulation of probiotic strains exclusively as vaccine and drug delivery vehicles are promising and rapidly growing area of research ^{1,15}.

The yeast *S. boulardii* can be considered as a candidate probiotic for future engineering. To facilitate the genetic manipulation of this yeast, we used the classical UV mutagenesis to produce uracil auxotroph mutants of *S. boulardii* as a host for recombinant protein production. The UV dose-response curve demonstrated a 90% killing rate after 23 s of UV irradiation. This result is in agreement with the time range reported by Hashimoto *et al* (20-40S) ⁷.

Ura3⁻ mutants were selected on 5-FOA plates. 5-FOA is toxic to yeast cells that can synthesize the *ura3* gene product, and therefore makes them unable to grow on 5-FOA-containing media ¹¹. In addition to act as a posi-

tive selection marker, the *URA3* gene can also be used for the negative selection (counter selection). In this regard, the presence of *URA3* confers sensitivity to FOA, while *ura3*⁻ negative cells are FOA resistant. This concept has been used in designing the *ura*-blaster gene constructs as a tool in multiple gene disruption experiments in *S. cerevisiae* ¹⁶. Hence, the generation of uracil auxotrophs of *S. boulardii* provides an opportunity for gene deletion studies in this organism.

To complement the *ura3*⁻ phenotype, the *URA3* gene was amplified from the *S. boulardii* genome and cloned into pGEM-Teasy vector. The restriction analysis of the *URA3* fragment from *S. boulardii* showed a pattern identical to its homologue in *S. cerevisiae*. This pattern was expected as the analysis of sequence data from different strains of *S. boulardii* had confirmed a high similarity between *S. boulardii* and *S. cerevisiae* in DNA level ¹⁷.

Bile and acid resistance are the most important prerequisites for probiotics to stay alive in the digestive tract of their hosts. Among the three isolated *ura3*⁻ mutants, only one (*S. boulardii* M2) showed acceptable resistance to acid and bile. Similarly, Sharaf *et al.* have used EMS mutagenesis and inter-specific protoplast fusion to isolate improved probiotic yeasts. They isolated an adenine auxotroph mutant of *S. boulardii* with high tolerance to bile salt ¹⁸. Abosereh *et al* have also isolated highly resistant *S. boulardii* strains through protoplast fusion ¹⁹. The acid and bile resistance capability of mutants provide these strains with an advantage *in vivo*. Further *in vivo* studies are underway to evaluate other probiotic features of the mutant.

Conclusion

A uracil auxotroph mutant of the probiotic yeast, *S. boulardii*, was generated in this study. The mutant was complemented by *URA3* carrying constructs, confirming the inactivation of this gene in the mutant. Bile and acid resistance of the mutant was the same as wild type strain. This mutant can be used as a

probiotic host for the *in vivo* production and delivery of various recombinant products to the GI tract.

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