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Genetic Improvement of *Saccharomyces boulardii* R7 and Generate Suitable Strains for Synthesis and Expression of Recombinant Products

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHAJ and AAS designed the study and wrote the protocol. Author RAZ wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Saccharomyces boulardii R7 was mutagenized using UV radiation. Uracil auxotroph mutants were isolated and four of these mutants showed stable mutation. Two mutants *Sb*R7M7 and *Sb*R7M10 were chose for transformation according to their similar behavior with wild type (*Sb*R7) isolate. The two mutants (*Sb*R7M7 and *Sb*R7M10) were transformed with pYES2, which was extracted from *Escherichia coli* Top10, and two transformants (*Sb*R7T7, *Sb*R7T10) obtained. The transformation was confirmed by isolating the plasmid (pYES2) from these transformants and used to transform *E. coli* Top10 (free of plasmid). Some probiotic properties were studied for the two transformants (*Sb*R7T7, *Sb*R7T10) compared with *Sb*R7. They showed a noticed improvement in autoaggregation ability, an improvement of antagonistic activity toward *E. coli:* O157:H7 and *Candida albicans* and they reduced cholesterol ratio after 24 hr of incubation, however, the ratio increased after 48 hr of incubation.

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1. INTRODUCTION

Saccharomyces boulardii is a tropical strain of yeast that is thermophilic and mostly non-pathogenic to humans [1,2]. It was first isolated from the skin of lychee and mangosteen fruits in 1923 by the French scientist Henri Boulard in the Indo-China region, and has since then shown to be effective treatment for diarrhea both in humans and animals and other gastrointestinal disorders caused by the administration of antibiotics [3,4,5]. *S. boulardii* is a well known probiotic yeast, which is used alone or in combination with probiotic bacteria to support digestive system [6,7]. In genetic manipulation methods, the selection of recombinants is usually performed by employing a suitable selection marker on a plasmid carrying the gene encoded that marker. In probiotic applications the antibiotic resistance markers are considered as a major concern. Hence, it is necessary to remove the antibiotic resistant gene from the host prior to commercial application [8]. The auxtrophic markers may be a better substitute and commonly used practically, but they require appropriate host strains which are auxotrophic for the specific nutrients corresponding to the inactivated gene [9]. The *URA3* gene is one of theses markers that encodes orotidine 5-monophosphate decarboxylase (OMPDCase), an enzyme involved in the *de novo* synthesis of pyrimidine ribonucleotides. The inactivation of *URA3* results in uracil auxotrophy and 5-fluoroorotic acid resistance phenotype [10]. Only recently, an auxotrophic strains of the probiotic yeast have been developed [11,12]. There remains a need to generate auxotrophic strains of *S. boulardii* that can be easily manipulated without the use of antibiotic resistance markers. Such an auxotrophic strain would make *S. boulardii* a much safer and more efficient host to express and deliver recombinant proteins to treat gastrointestinal disorders [12]. Although *S. boulardii* has been widely studied due to its probiotic properties against several gastrointestinal tract disorders, very few studies evaluated the use of this yeast as a vehicle for expression of foreign genes of interest with biotechnological applications [13,14].

According to the great importance of probiotic yeast, many studies interested in genetic improvement of *S. buolardii* in order to develop their desired and appropriate characteristics. The present study aimed to isolate uracil auxotroph mutants of *S. boulardii* through UV mutagenesis. The auxotroph mutants were transformed by pYES2 plasmid (harboring the URA3 gene). The mutants could be a suitable probiotic host to accept various genes, therefore, expression and production of recombinant products.

2. MATERIALS AND METHODS

2.1 Microorganisms and Media

Saccharomyces boulardii R7 was isolated and identified in a previous study, and showed good physiological and probiotic properties [15]. This yeast isolate obtained from the College of Agriculture at University of Baghdad. The isolate was maintained at 4℃ on YPD (2% agar, 1% yeast extract, 2% peptone, 2% glucose/dextrose in distilled water) slants, YPD broth was used for the activation of this yeast. *E. coli* TOP10 harboring pYES2 plasmid and the same strain free of this plasmid (Invitrogen) were maintained at 4℃ on Luria-Bertani (Sigma) slants.

Yeast Nitrogen Base (YNB; Sigma) was prepared at a concentration of 0.67% and supplemented with 2% glucose, 10 mM uridine and uracil, and 0.1% 5-FOA (Sigma) to use in screening of auxotrophs.

2.2 Mutagenesis and Isolation of Mutants

Saccharomyces boulardii R7 was mutagenized using UV radiation depending on Hamedi et al. [11]. The tray for irradiation was 15×25 cm, which exposed sample in a glass Petri dish, to direct irradiation from a bulb (Philips, TUV 15 W/G15) and the distance between the UV source, and irradiated suspension was 20 cm. SbR7 isolate was first grown in YPD broth at 37℃ for 20 hrs, and then 10 ml of yeast culture was harvested in sterile phosphate buffer (pH 7) and mixed with a vortex mixer, followed by centrifugation at 4000 rpm for 10 min. The cell pellet was resuspended in phosphate buffer (20 ml) and the suspension (20×10^7 cells/ml) was poured into sterilized Petri dishes and exposed to UV radiation under sterile conditions with gently agitated by magnetic stirrer. Then samples (0.5 ml) of cell suspension were taken at 10 sec intervals over a 100 sec period. Irradiated cell suspensions were stored in foil-wrapped tubes at 4℃ overnight to avoid photoreactivation, diluted to appropriate dilution and plated on YPD agar (2

replicates per dilution). Plates were incubated at 37℃ for 48 hours to determine the viable count and survivals of yeast cells. According to the survival curve, the treatment that leads to a survival percentage of approximately 10% was considered to have a higher mutation rate. From this treatment, samples were spread onto YNB-FOA plates and incubated at 37℃ for one week to select for cells lacking a functional URA3 gene. Colonies resistant to 5-FOA were screened by multiple restreaking onto plates supplemented with uracil and uridine (YNB-UU) and without supplements (YNB). Uracil auxotroph mutants were detected by their ability to grow only in the presence of this component.

These mutants were tested for mutation reversion by inoculating 10 ml of YPD broth, incubated at 37℃ for 20 hrs, followed by centrifugation (4000 rpm) at 4℃ for 10 min. The cell pellet was washed and resuspended in 1 ml of phosphate buffer (pH 7). Samples (0.1 ml) from appropriate dilutions were spread on YNB-UU and YNB plates and incubated at 37℃ for 5-10 days. The colonies grown on YNB considered as revertants and the frequency of revertants was calculated as the following equation:

Frequency of Uracil Revertant = (Uracil Revertants / Total viable count) × 100

2.3 Physiological and Probiotic Characteristics

Some physiological characteristics were investigated for auxotroph mutants compared with wild type. Growth at different temperatures, Bile salt resistance assay [16] and low pH resistance assay [17].

In addition some probiotic properties were studied for transformants compared with wild type. Autoaggregation ability [18], antagonistic ability of cells and their filtrate [19,20] and reduction of cholesterol [21].

2.4 Transformation of Uracil Mutants

The plasmid pYES2 (containing URA3 gene of *S. cerevisiae*) was extracted from *E. coli* TOP10 using DNA-spin^{TM} plasmid DNA Purification Kit (iNtRON BIOTECHNOLOGY). Competent cells of uracil auxotroph mutants were obtained according to Kawai et al. [22], then transformed with this plasmid using electroporation method by Gene pulser® Cuvette (Invitrogen). Following the transformation, cells were plated on YNB agar medium lacking uracil and uridine supplements. The plasmid (pYES2) was isolated from the transformants and also used to transform *E. coli* Top 10 cells (free of plasmid). The plasmid was isolated again from this bacterium to confirm the transformation.

3. RESULTS

3.1 Mutagenesis of *S. boulardii* R7 (SbR7)

3.1.1 Survival curve

The results (Fig. 1) demonstrated that the percentages of survival of *Sb*R7 cells were decreased by increasing the exposure time of UV radiation. The killing percentage was 100% at exposure time 50 sec., while the UV exposure time required to kill 90% of *Sb*R7 cells was 25 sec. Therefore, this treatment (25 sec.) was used to looking for uracil auxotroph mutants.

Auxotroph mutants were isolated from this treatment (25 sec). The results showed that five uracil auxotrophic mutants were obtained (They were able to grow on YNB-UU medium, but not on YNB) (Fig. 2).

The mutants were tested for mutation reversion, it was found that one mutant (*Sb*R7M4) reverts in frequency of 1.4×10^{-5} and four mutants (*Sb*R7M1, *Sb*R7M7, *Sb*R7M10 and *Sb*R7M11) showed stable mutation during 10 days of incubation.

Some physiological characteristics were investigated for uracil auxotroph mutants compared with wild type. The results indicated that *Sb*R7M7 and *Sb*R7M10 show similar to wild type growth characteristics (Fig. 3), low pH resistance (Table 1) and bile salt resistance (Table 2). Therefore, these (two) mutants were selected for transformation experiment.

3.2 Transformation

The plasmid pYES2 was extracted from *E. coli* Top 10 and digested with either *DraI* or *EcoRV* restriction enzymes, to confirm that the pYES2 construct is present intact (Fig. 4). The two uracil auxotrophic mutants (*Sb*R7M7 and *Sb*R7M10) were transformed with this plasmid. The results indicated that transformants of both mutants were obtained. They were able to grow on YNB

medium and this mean that these colonies were transformed with pYES2. One transformant for each mutant was selected and termed as *Sb*R7T7 and *Sb*R7T10. To confirm the transformation, the pYES2 plasmid was isolated from *Sb*R7T7 transformant. The results (Fig. 5) showed that *Sb*R7T7 contain the plasmid and this plasmid was also used to transform *E. coli Top10* cells (free of plasmid). Transformants (*E. coli*) were selected according to ampicillin resistance on LB medium supplemented with ampicillin. The plasmid was isolated again from this bacterial transformants to confirm the transformation (Fig. 5).

In addition to the low pH and bile salt resistance capability of the mutants, which provide these mutants with an advantage *in vivo*, other probiotic features were detected for transformants (*Sb*R7T7, *Sb*R7T10) which included the autoaggregation ability, the antagonistic ability of cells and their filtrate and reduction of cholesterol. The two transformants showed that there was a noticed improvement in autoaggregation ability, which was 69.72 and 68.95% compared with 65.37% for wild type. The results also showed an improvement of antagonistic activity of the two transformants toward *E. coli* O157:H7 and *C. albicans.*

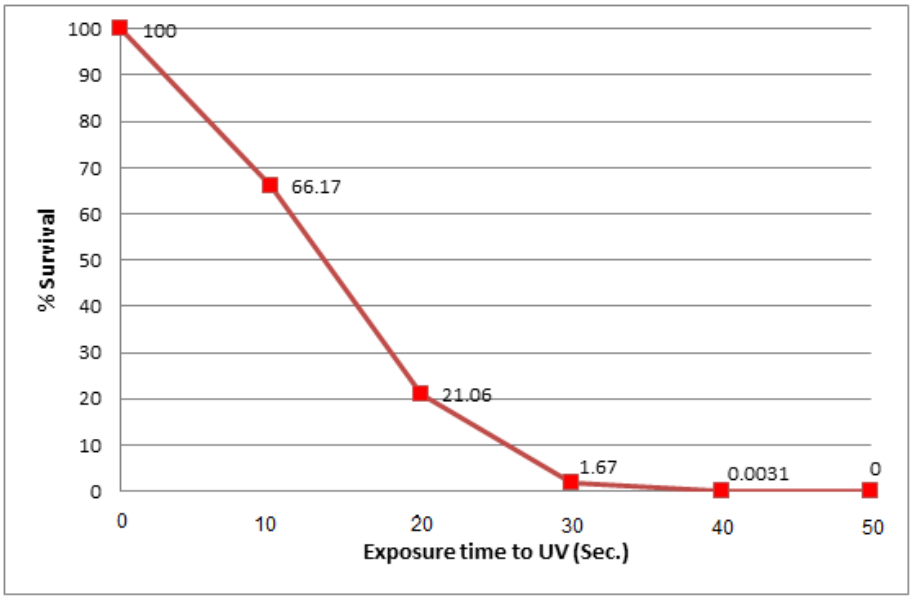

Fig. 1. Survival curve of *S. boulardii* R7 exposing to UV radiation

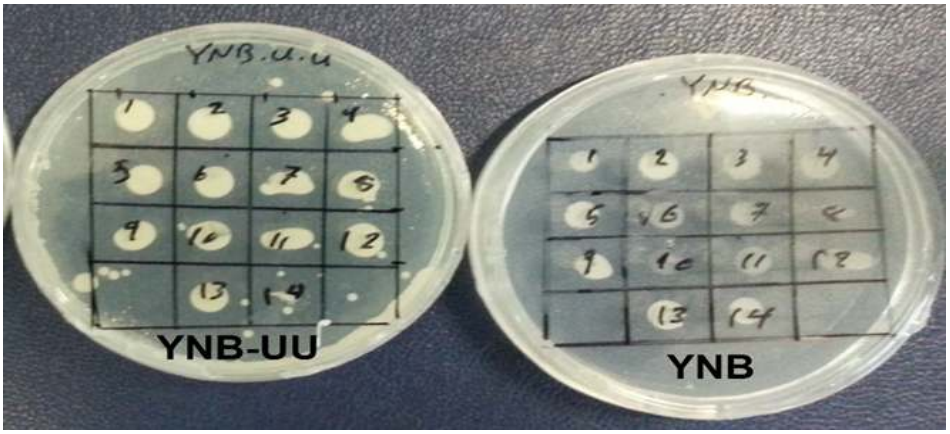

Fig. 2. Uracil auxotroph mutant colonies on YNB plates with and without uracil

The diameter of inhibition zone caused by *Sb*R7T7 was 20 and 21 millimeters, respectively, while that caused by *Sb*R7T10 was 19 and 21 millimeters, respectively, compared with an inhibition zone for wild type, which was 18 and 19 millimeters respectively. In addition, cell filtrates for both transforments showed best inhibition of *C. albicans* when compared with wild type.

The transformants (*Sb*R7T7 and *Sb*R7T10) were reduced cholesterol ratio to 27.78 and 26.42% respectively after incubation period for 24 hr and these percentages increased to 48.72 and 52.86% after 48 hr incubation period when compared with wild type, which reduced 25.59% and 53.90 of cholesterol in the same incubation periods.

Table 1. Viable counts of wild type and uracil auxotroph mutants in low pH for 1, 2 and 3 hours

pH	Incuba / Isolate	0 hr.	1 hr.		2 hr.		3 hr.	
		10^7 CF U/ml	10^7 CF U/ml	% Survival	10^7 CF U/ml	% Survival	10^7 CF U/ml	% Survival
3	SbR7WT	97	97	100	95	97.9	93	95.8
	SbR7M7	94	94	100	90	95.7	89	94.06
	SbR7M10	102	102	100	100	98.0	95	93.13
	SbR7M11	90	90	100	87	96.6	81	90.00
	SbR7M14	92	92	100	89	96.7	83	90.21
2	SbR7WT	97	85	87.6	45	46.4	19	19.6
	SbR7M7	94	80	85.1	40	42.5	14	14.8
	SbR7M10	102	86	84.3	46	45.0	20	19.6
	SbR7M11	90	72	80.0	36	40.0	11	12.2
	SbR7M14	92	76	82.6	35	38.0	11	11.9
1	SbR7WT	97	19.9	20.5	0.0074	0.00007	0.00027	0.0000027
	SbR7M7	94	14.8	15.7	0.0062	0.00006	0.00022	0.0000023
	SbR7M10	102	17	16.6	0.0079	0.00007	0.00029	0.0000028
	SbR7M11	90	6.8	09.5	0.0038	0.00004	0.00012	0.0000013
	SbR7M14	92	6.2	06.8	0.0027	0.00003	0.00014	0.0000015

** Each No. represents the mean of duplicates*

Table 2. Viable counts of wild type and uracil auxotroph mutants in different concentrations of bile salt for 1, 2 and 3 hours

% bile salt	Incubati / Isolate	0 hr.	1 hr.		2 hr.		3 hr.	
		10^7 CF U/ml	10^7 CF U/ml	% Survival	10^7 CF U/ml	% Survival	10^7 CF U/ml	% Survival
1	SbR7WT	95	95	100	93	97.8	78	82.1
	SbR7M7	94	94	100	93	98.9	79	84.0
	SbR7M10	100	100	100	97	97.0	80	80.0
	SbR7M11	92	92	100	88	95.6	71	77.1
	SbR7M14	93	93	100	89	95.6	74	79.5
2	SbR7WT	95	91	95.7	66	69.5	41	43.1
	SbR7M7	94	89	94.6	63	67.0	38	40.4
	SbR7M10	100	96	96.0	69	69.0	42	42.0
	SbR7M11	92	86	93.4	60	65.2	35	38.0
	SbR7M14	93	85	91.3	57	61.2	32	34.4
3	SbR7WT	95	50	52.6	29	30.5	2.2	2.31
	SbR7M7	94	47	50.0	26	27.6	1.8	1.91
	SbR7M10	100	53	53.0	28	28.0	2.3	2.30
	SbR7M11	92	27	29.3	2.6	02.8	NG	0.00
	SbR7M14	93	29	31.1	3.8	04.0	NG	0.00

** Each No. represents the mean of duplicates.*

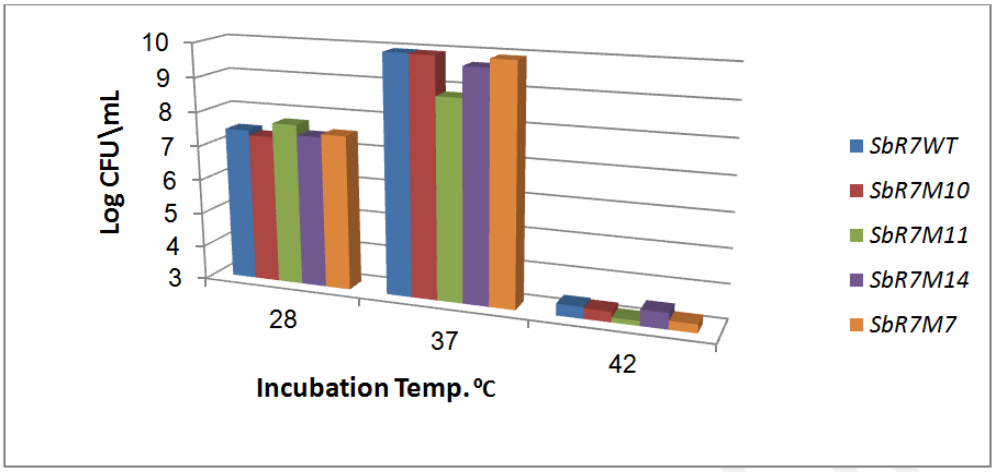

Fig. 3. Uracil auxotroph mutants and wild type grown at different temperatures

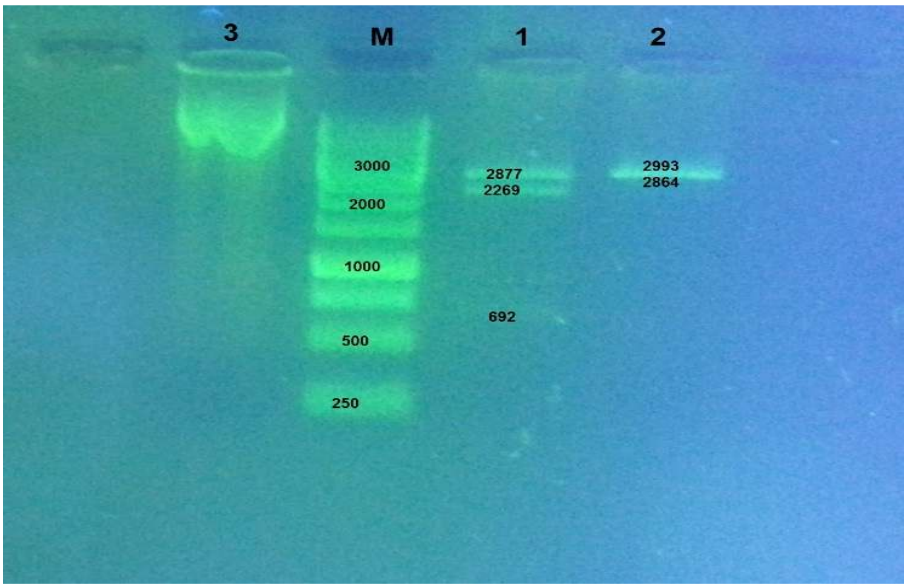

Fig. 4. Gel electrophoresis for plasmid isolation from *E. coli* Top10. Electrophoresis was performed on 2% agarose gel with 7V/cm for 1.5 hr. Lane1: *DraI* digested plasmid. Lane 2: *EcoRV*digested plasmid. Lane 3: undigested plasmid (pYES2). Lane M: DNA markers (250-3000 bp)

4. DISCUSSION

In an attempt to improve *S. boulardii* strains that could be suitable for synthesis and delivery of recombinant proteins to the gastrointestinal tract, UV mutagenesis was employed to generate uracil auxotroph mutants of *S. boulardii* as a host for recombinant proteins. The survival curve indicated a 90% killing rate at 25 sec. of UV irradiation. This result is in agreement with Hashimoto et al. [8] and Hamedi et al. [11] who reported that a 90% killing rate after (20-40 sec) and 23 sec of UV irradiation respectively.

Five uracil auxotrophic mutants were obtained and four of them showed stable mutation. This means that the mutation in the uracil gene of these mutants is steady and stable. It was mentioned that stable auxotroph mutant occurs in a uracil gene (s) if there is no revertant appeared during incubation on YNB medium [11,23].

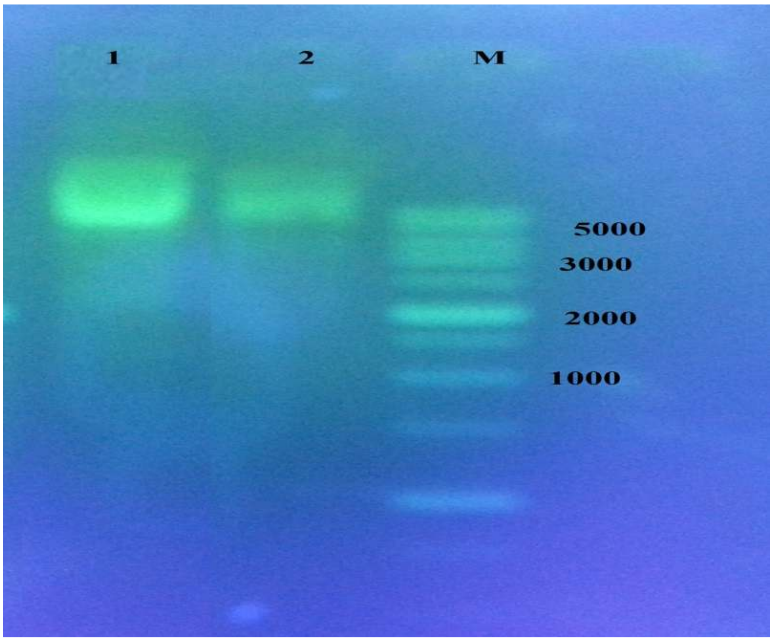

Fig. 5. Gel electrophoresis for pYES2 isolation. Electrophoresis was performed on 0.8% agarose gel with 7V/cm for 1.5 hr. Lane1: pYES2 isolated from *Sb*R7T7. Lane 2: pYES2 isolated from *E. coli* Top10 transformant. Lane M: DNA marker

Only two mutants (*Sb*R7M7 and *Sb*R7M10) showed similar characteristics (growth, low pH and bile salt resistance) with wild type. Bile salt and low pH resistance are the most important traits which required for microorganisms used as probiotics to enable them to resist extreme conditions [24]. Similarly, UV irradiation was used to generate uracil auxotroph mutants of *S. boulardii*. Hudson et al. [12] isolated three uracil auxotrophic mutants that show a low rate of reversion to wild type growth. Hamedi et al. [11] also isolated three uracil (URA3) auxotroph mutants, these mutants were able to grow normally in the absence of uracil, when the URA3 gene was introduced. In vitro analysis showed that the acid and bile resistant capacity of one of these mutants is similar to the wild type. Sharaf et al. [25] have isolated an adenine auxotroph mutant of *S. boulardii* with high tolerance to bile salt using Ethyl Methyl Sulphonate mutagenesis and interspecific protoplast fusion. Highly resistant *S. boulardii* strains to low pH and bile salt were also isolated by using protoplast fusion [26].

nI this research, we used a commercially kit for preparation of competent cells and transformation of *S. cerevisiae*. Since *S. boulardii* genome is not sequenced, the transformation of this probiotic yeast has so far relied in the available genetic tools available for the highly related species *S. cerevisiae*. The two uracil auxotroph mutants (*Sb*R7M7 and *Sb*R7M10) were transformed successfully with pYES2 plasmid. Transformants from both mutants were obtained and confirmed by isolating this plasmid from one transformant then using to transform *E. coli*. *E. coli* is a common host for the expression of various genes and production of proteins in industrial applications as well as the copy number of this plasmid in this bacterium is more than in yeasts [27]. Similarly, Douradinha et al. [13] successfully transformed *S. boulardii* ATCC MYA-796 with a plasmid DNA, pYC440, using the same kit, with minor modifications. They reported an efficient and rapid method to transform and subsequent screening of *S. boulardii* with plasmid DNA.

Some probiotic properties were studied for transformants (*Sb*R7T7, *Sb*R7T10). They showed that their ability to reduce cholesterol is approximately similar to wild type, however, an improvement in autoaggregation ability and antagonistic ability were detected. These properties may render these mutants a very good candidate for recombinant proteins and drug delivery to treat gastrointestinal disorders.

The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Structural genes corresponding to virtually any genetic trait can be identified by complementation from plasmid libraries [23]. Hudson et al. [12] reported that *S. boulardii* mutants (ura3) possess all the characteristics needed for safe and efficient use as an oral drug delivery system. These mutants can be transformed and selected using auxotrophic markers to avoid reliance on antibiotic selection.

5. CONCLUSIONS

The obtained auxotrophic mutants showed similar characteristics (growth, low pH and bile salt resistance and their ability to reduce cholesterol) with wild type. However, they showed also an improvement in autoaggregation ability and antagonistic ability. Accordingly, these mutants may be efficient candidates of the probiotic yeast (*S. boulardii*) for synthesis, expression and delivery of proteins with therapeutic application.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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