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Mutation Induction for Genetic Improvement of *Saccharomyces boulardii* Which Used as Probiotic Yeast

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Abstract: *Saccharomyces boulardii* was used to examine the changes in two important points; the ability to tolerate 3%Bile salts and the antimicrobial activity on some harmful indicator microorganisms; following Gamma (γ) irradiation. Induction of mutation in *S. boulardii* was carried out by 1,2,3,4 and 5 KGy exposure of γ irradiation. Results revealed that the survival percentages were decreased by increasing the doses of γ rays whereas the survival percentage was 2.67% at exposure dose 5KGy. On the other hand, the mutant percentages were increased by increasing the radiation intensities, i.e., doses. The highest numbers of mutants were induced as a result of 4KGy dose of γ rays applications, which gave the highest mutants percentage (14.29%). Seven mutants were isolated as auxotrophes and their nutritional requirements were determined. *Saccharomyces boulardii* and their resulted mutants were tested for 3% Bile salts tolerance. The results showed that mutant No.Sb.M4 was the highest while mutants No.Sb.M6 and Sb.M7 were the lowest. On the other hand, Mutants No.Sb.M2 and Sb.M5 were similar to *S.boulardii* (W.T). The antimicrobial effect of *S. boulardii* and three mutants, Sb.M2, Sb.M4 and Sb.M5 against the pathogenic microbes *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus or Bacillus cereus* was determined. No antimicrobial effect was scored with *S. boulardii* on all pathogenic tested strains. All mutants were also not able to give any antimicrobial effect on both *B. cereus* and *E. coli*, while the effect was pronounced on both *S. aureus* and *P. aeruginosa*.

Key words: Mutation, γ irradiation, Nutritional requirement, Bile salt tolerance, Antimicrobial activity.

INTRODUCTION

Many health claims have been made regarding probiotics role in preventing or curing gastrointestinal illnesses. Several studies have been found probiotic to be useful in treating some types of diarrhea[18].

Since *Saccharomyces boulardii* is a rich source of B vitamins and chromium, it has been studied extensively for its medicinal properties. The yeasts *Saccharomyces boulardii* and *Saccharomyces cerevisiae* (commercial baker's yeast) has been reported as a potential biotherpeutic agent for the treatment of microbes associated diarrhea and colitis[9].

Saccharomyces boulardii is safe, non toxic, non pathogenic, thermophilic yeast that is recognized to have probiotic effectiveness used alone and/or in combination with other probiotics to support digestion. It is useful as biotherpeutic agent in combination with standard antibiotic for the treatment of *Clostridiuni difficile* diarrhea and colitis. Diet supplementations with the probiotics *Lactobacilli* and *Saccharomyces boulardii* was reported to help in reducing some effects of aging[1,8,13].

Genetic improvement of *Saccharomyces* and other yeast strains has traditionally relied on random mutagenesis or classical breeding and genetic crossing of two strains followed by screening for mutant or fusants exhibiting enhanced properties of interest[25,22]. Recent development of sophisticated methods in the field of recombinant DNA technology has enabled us to manipulate given pathway of interest and hence to improve the cell by a more directed approach. Thus, it is now possible to introduce specific genetic perturbations in terms of modifying the promoter strength of a given gene, to perform gene deletions, or to introduce whole new genes or pathways into the cell[16,4].

The aim of this study was to obtain highly efficient biotherapeutic yeast strains by using Gamma irradiation by the genetic improvement of some probiotic *S.boulardii* important properties. The promising strains in this study could be used in many medical applications and for successive genetic improvement as well.

MATERIALS AND METHODS

Microbial strains and culture condition: Pure culture of *Saccharomyces boulardii* used in this study was kindly obtained from Microbial Genetics Dept., NRC, Dokki, Giza, Egypt. The yeast was maintained as slant of YEP agar medium as a complete medium (CM), Santangelo[19]. When required, the yeast culture was activated by

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transferring to YEP plates, incubated for 48 h at 30 ^{O}C. The cells were grown before irradiation up to the stationary phase of growth on a solid growth medium for 4 days. After attaining the stationary phase of growth, the cells were washed twice with buffered saline (0.8% NaCl, pH 6) and an initial cell concentration of 5 x 10^7 cells / ml determined by the method of[9] was prepared. Minimal medium (MM) was used for the isolation of yeast mutants, i.e., auxotrophic cells[21].

The indicator bacteria used for antimicrobial activity were as follows: *Escherichia coli* ATCC 69337, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 20231and *Bacillus cereus* ATCC 33018. These strains were obtained from Egyptian Microbial Culture Collection (EMCC) at Cairo, Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University. Cultures of all indicator bacteria were propagated at 37 ˚C/ 24 h and maintained on nutrient agar.

Gamma (γ) mutagenesis: Gamma (γ) irradiation as a physical mutagenic agent was used with *S. boulardii* for induction of highly efficient biotherapeutic yeast strains. Mutagenesis of yeast strain was carried out according to[15] at National Centre for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt, using different radiation intensities, i.e., different doses from irradiation source (1, 2, 3, 4 and 5 KGy). Cultures were irradiated in air at ambient temperature and atmosphere pressure with ^{60}Co as a source of Gamma with a rate of 302KGy/ h. *S. boulardii* cells were grown in YEP broth medium with shaking at 30^{0}C.Cells then were harvested during the stationary phase of growth and washed twice as above. The washed cells were resuspended in the buffered saline. The cell suspension of the wild type of *Saccharomyces boulardii* in the buffer solution was treated with Gamma rays as mentioned previously with gentle agitation. To select yeast mutants, a serial dilution was done from each treatment and portions of 0.1 ml of suitable dilutions of yeast suspension were spread onto the surface of plates containing the CM. Plates were then incubated for 48 h at 30^{0}C. Colonies developed after incubation were counted, picked up and transplanted on both MM and CM media. The colonies which showed growth on CM but not on MM were considered as auxotrophes (mutants). Survival and mutation percentages were estimated for each treatment.

Nutritional requirements of yeast mutants: Nutritional requirements of each mutant was identified by replica plating method on the MM supplemented with one or more than each of the amino acid (250 mg/L), vitamins(2.5 mg/L) and nitrogen bases (25 mg/L) according to[17,15]. The plates were incubated for 3 days at 30^{0}C. Nutritionally deficiently yeast mutant was detected by its ability to grow on MM supplemented with a certain nutrient and inability to grow in its absence. The selected mutants which have one requirement were grown on minimal broth medium /24 hrs as a mean of starvation. All possible pair wise combinations were then tested for their growth on solid MM in order to find out the functional relationships (concerning growth requirements) between them.Mutants which shows complementation and those which do not complement each other were recorded after incubation for 24 h at 30^{0}C.

Bile salt tolerance: Culture of *S. boulardii* (w.T) and its resulted mutants were evaluated for viability and growth in YEP broth supplemented with 0.2% sodium thioglycolate and 3%Bile salts (Oxoid) according to[5]. The culture were plated on YEP agar using a pour plate method. All strains were incubated for48 h at 30˚C. After incubation, colony forming units (CFU) were counted and recorded.

Antimicrobial activity: The antimicrobial activity of the cell-free supernatants of the examined yeast strains against the indicator microorganisms was determined using agar diffusion well assay[23] but without neutralization.

RESULTS AND DISCUSSIONS

Gamma (γ) mutagenesis: Gamma irradiation, as a physical method, is known to cause injury to microorganisms and has been used widely to prevent or delay food spoilage[7]. Mutation as a result of gamma radiation was achieved by 1, 2, 3, 4 and 5 KGy at dose rate of 3.2 KGy/h (Table 1 and Fig.1).

Results in Table (1) and Fig. (1) showed that the percentages of survival and mutation were affected by γ doses. The survival percentages were decreased by increasing the doses of gamma radiation whereas the survival percentage was low (2.67%) at exposure dose (5KGy). On the other hand, the mutant percentages were increased by increasing the radiation intensities, i.e., doses up to 4 KGY. Data in table (1) also showed that the highest number of mutants was induced as a result of exposure dose (4KGy) which gave the highest mutant percentages (14.29%).

Nutritional requirements: *Saccharomyces boulardii* mutants obtained after γ irradiation treatments were tested for their requirements. These mutants were grown on MM supplemented with one or more of the amino acids, vitamins and nitrogen bases. Data in Table (2) show the nutritional requirements of yeast mutants.

Results in Table (2) showed that mutant No. Sb.M1, Sb.M2, Sb.M3, Sb.M4, Sb.M5 and Sb.M7 were found to be nutritionally deficient of Valine, adenine, Glycine, lysine, Arginine and Tryptophane, respectively. Only one mutant, SbM6, was found to require lysine and adenine for growth.

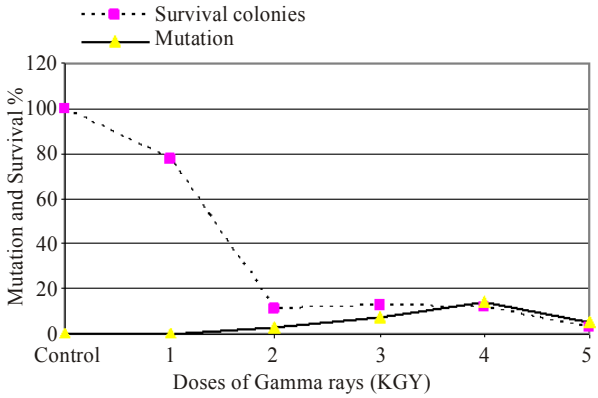

Fig. 1: Survival and mutant percentages of *S. boulardii* exposed to different doses of Gamma irradiation

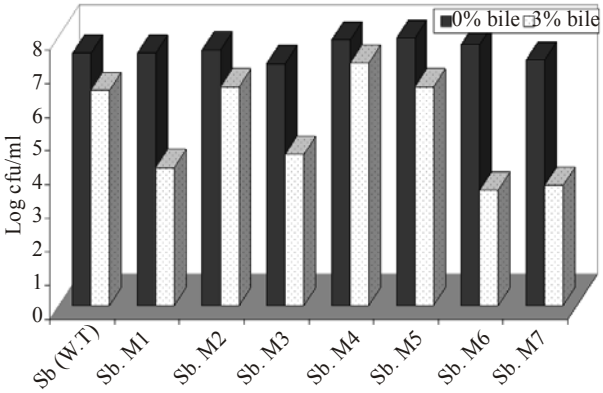

Fig. 2: Viability of *S. boulardii* and their mutants in presence of 3% Bile salt

Table 1: Effect of different doses of Gamma irradiation on survival and mutant percentages of *S. boulardii* strain.

Doses (KGY)	Survival colonies/ ml No.	%	No. of tested colonies	Mutants No.	%
0	600	100	100	0	0.00
1	465	77.5	69	0	0.00
2	426	11.00	31	1	3.22
3	78	13.00	28	2	7.15
4	73	12.17	21	3	14.29
5	16	2.67	18	1	5.56

Table 2: Nutritional requirements of *S. boulardii* auxotrophic mutants.

Yeast mutant strains	Doses of Gamma radiation	Requirements Amino acids	Nitrogen bases
Sb.M1	2	Valine	--
Sb.M2	3	--	Adenine
Sb.M3	3	Glycine	--
Sb.M4	4	Lysine	--
Sb.M5	4	Arginine	--
Sb.M6	4	Lysine	Adenine
Sb.M7	5	Tryptophane	--

Bile salt tolerance: The ability to grow (tolerance) in the presence of 3% Bile salt was varied among *S. boulardii* (w.T) and their resulted mutant strains (Fig.2). Data showed clearly that mutant Sb.M4 grew significantly

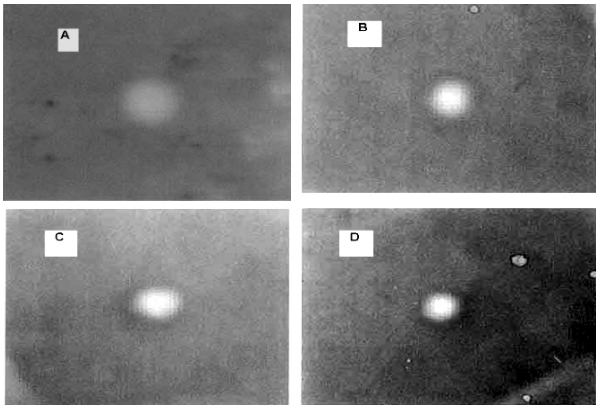

Fig. 3: The antibacterial effect of cell- free supernatants of *Saccharomyces boulardii*, SbM2, Sb.M4 and Sb.M5 on *P. aeruginosa*.
A: The antibacterial effect of *Saccharomyces boulardii* on *Pseudomonas aeruginosa*
B: The antibacterial effect of mutant No. Sb.M2 on *P. aeruginosa*
C: The antibacterial effect of mutant No. Sb.M4 on *P. aeruginosa*
D: The antibacterial effect of mutant No. Sb.M5 on *P. aeruginosa*

Table 3: The antimicrobial effect of cell- free supernatants of *Saccharomyces boulardii*, Sb.M2, Sb.M4 and Sb.M5 on various indicator bacteria.

Yeast strains	Inhibition zone diameter in mm of indicator bacteria *E. coli*	*S. aureus*	*P.aeruginosa*	*B. cereus*
S. boulardii	-	-	-	-
SbM2	-	8	8	-
SbM4	-	-	10	-
SbM5	-	6	8	-

better than the other mutants. Mutant strains no. Sb.M2 and Sb.M5 were similar to *S.boulardii* (W.T) while mutant strains No.Sb.M6 and Sb.M7 were exhibiting significantly less growth than all other mutants. Although the Bile salt concentration of the human G1 tract varies, the mean intestinal Bile concentration is believed to be 0.3%,[10]. This concentration of Bile has consequently been used in most studies screening for Bile resistant strains[11,3].

This property may provide these strains with an advantage *in vivo*. These results are generally in harmony with those reported by[2] who indicated that *Saccharomyces boulardii* displayed good resistance to 0.3%.

Antimicrobial activity: The ability of cell – free supernatants of *Saccharomyces boulardii* W.T strain and three mutants (Sb.M2,Sb.M4 and Sb.M5) to retard or suppress the growth of some harmful indicator bacteria is presented in Table (3) and Fig. (3).

Results in Table(3) revealed that in case of *E. coli* and *B. cereus*,no improvements as antimicrobial activity was found of all mutants, while in the cases of *S. aureus* and *P. aeruginosa*, positive improvements were obtained.

Results in Table (3) also showed that *S. boulardii* wild type strain was not able to give any antimicrobial activity on *E. coli* and *P.aeruginosa* and this result is in agreement with those obtained by[2]. They revealed that *S. boulardii* was not able to give any antibacterial effect against *E. coli* growth. The highest clear zone diameter was achieved by mutant no. Sb.M4 against *P.aeruginosa* (10mm) while the lowest was achieved by mutant no. Sb.M5 against *S. aureus* (6mm). Fig. 3 (A, B, C and D) shows the inhibition zone (clear zone) due to the antimicrobial effect of the three mutants (Sb.M2, Sb.M4 and Sb.M5) on *P.aeruginosa* in comparison with *S. boulardii* wild type strain.

The action of ionizing radiation on living cell is determined by both the physical properties of the ionizing radiation and the biological ability of cells to recover from potentially effective damage[24]. In this study the survival curves is obtained for a number of yeast cells with different radio sensitivity after a low and high gamma radiation. Several possible explanations have been widely discussed for the slower recovery observed when Gamma rays increased, e.g., the primary radiation damage is more severe and complex and therefore required a long time to be repaired[12] and this is an interesting hypothesis, likely to be true. The number of small and intermediate size DNA fragments is larger and a longer time is needed for repairs and recovery[14].

It is more likely that the repair systems involved are overwhelmed by the presence of more complex DNA damage has already suggested[6]. It is known that several DNA repair pathways are involved in the recovery from radiation damage. Among these, in stationary phase diploid yeast cells, homologues recombination is the pathway that is most frequently employed[20]. It can be concluded on this basis that the recovery process itself is not damage after densely ionizing radiation and high radiosensitive of mutant cells may also be related to the increased yield of the irreversible damage.

From the present results, it is concluded that the high yielding mutants having a highest effect on some pathogenic bacteria and also tolerate 3% Bile salts are highly recommended for various medical applications and for successive genetic improvement as well.

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