Molecular Analysis of Genetically Improved Therapeutic *Saccharomyces Cerevisiae* Strains with High Selenium Uptake

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Abstract: This study was initiated to take advantages of mutagenesis in conjunction with protoplast fusion technique to obtain hyper- polyploidy strains with high selenium uptake. After UV-mutagenesis, results showed that five mutants uptake nearly all selenium present in growing medium and six mutants uptake 3.5 fold of selenium than the original strain. Moreover, selenium uptake of superior yeast mutants in the presence of selenium oxide (100 mg/L) showed that yeast cells tolerate high selenium concentration in the growing medium and uptake percentages ranging from 46.84 to 79.38. Intra-specific protoplast fusion technique was carried out in order to obtain polyploidy isolates with high selenium uptake. Data showed that 38 fusants obtained from cross 1 and 36 fusants from cross 2. In cross No.1, 71 % of fusants uptake more selenium than the first parent (mutant 5/8) and 97.4% than the second parent (mutant 5/9). For cross 2, 36.9 % of fusants uptake more selenium than the first parent (5/8) and 100 % than the second parent (20/1). Finally, an attempt was done to evaluate the genetic effect of UV-mutagenesis and protoplast fusion on nucleotide sequence by random amplified polymorphic DNA (RAPD) analysis. Many differences were noticed in mutant and fusant strains in comparison the wild type strains. These differences in RAPD profiles confirmed the evidence of genetic variations in yeast genome after mutagenesis and protoplast fusion technique. Also, these differences could be used as genetic markers for genetic diversity of selenium uptake characteristics. [Journal of American Science. 2011;7(1):45-56]. (ISSN: 1545-1003).

Key words: Selenium uptake, UV-mutagenesis, protoplast fusion and RAPD

1. Introduction

Living organisms possess genetic responses to chemical stress triggered by a variety of chemical inducers, involving the expression of chemical metabolizing and detoxifying enzymes and antioxidants (Azevedo *et al*., 2003). These inducible responses are important to preserve cellular integrity and have been a focus of attention in the chemoprevention of mammalian carcinogenesis (Talalay *et at.*, 1995). A number of epidemiologic, experimental studies and clinical intervention trials have indicated that the micronutrient selenium has potential anticarcinogenic effects (Clark *et al*., 1996 and El-Bayoumy, 2001).

Selenium is an essential trace nutrient and selenium deficiency diseases are well known in veterinary medicine. Above trace levels, ingested selenium is toxic to animals and may be toxic to humans (Melwanki and Seetharmappa, 2000). Selenium tends to weaken the toxic action of some heavy metals in animals and human organisms (Stabnikova *et al.,* 2005).

Under appropriate conditions, yeasts are capable of accumulating large amounts of selenium, and incorporating them into organic selenium-containing compounds, mainly selenomethionine, which is the best source of selenium for organisms (Demirci *et al*., 1999; Suhajda *et al*., 2000; Schrauzer, 2001 and Petrera *et al.*, 2009).

This study is aimed to genetic improvement and construction of some therapeutic yeast. To achieve this goal, UV-mutagenesis and intra-specific protoplast fusion were carried out. Moreover, the genetic effect of UV-mutagenesis and protoplast fusion on nucleotide sequence by random amplified polymorphic DNA (RAPD) analysis is also considered.

2.Materials and Methods

A- Yeast strains

Saccharomyces cerevisiae NRRL Y-139, *Candida utilis* NRRL Y-184 , *Kluyveromyes marxianus* NRRL Y-2415 and UV-mutants isolated after different exposure times were used in this study

B- Growth media and reagents

1-YEPD medium: Stocks were maintained on this medium which consisting of 1% Bacto-yeast extract, 2% dextrose and 1.5% agar in distilled water.

2-Selenium (IV) oxide stock solution was prepared by dissolving 1 g selenium in 10 ml of hot distilled water and filter-sterilized .

3- Protoplast medium (Khattab, 1997)

Sacharomyces cerevisiae protoplasting medium containing (g/L): Malt extract, 3; glucose , 50; yeast extract, 3 and peptone , 5. The intial pH of the medium was maintained between 6.5 and 6.8 with 1M Na**O**H solution.

4-Regeneration medium (Takagi *et al* ., 1983): It was used to isolate tetraploid clones after protoplast fusion . It consists of (g/L) yeast extract, 1; peptone, 1; glucose , 20; KH_2 PO_4 , 1.5; $MgSO_4$. $7H_2O$, 1 and sorbitol, 182.2.

5- Protoplast fusion buffers:

5.1. Pre- treatment solution (Spencer *et al.*, 1980). It consist of EDTA- Na_2 pH 7.6, 20 mM ; Tris-HCl pH 7.6 200 mM and 2-mercaptoethanol, 100 mM.

5.2. Protoplasting buffer (Spencer *et al.*, 1980): It consists of KH_2 PO_4, 80 mM; sodium citrate, 16 mM and KCl, 600 mM . The final pH of this buffer was 5.8.

5.3. Polyethylene glycol (PEG) solution (Farahanak, *et al*., 1986): It consists of PEG (MW 3350), 35 %(w/v); $CaCl_2$, 10 mM and sorbitol, 800 mM.

5.4.

C- Growth conditions

Yeast strains and UV- mutants were grown in YEP broth (50ml) at 30^{0}C with shaking (150 rpm) .

D- Evaluation of mutants for selenium uptake

I-Screening of superior mutants

Selenium (IV) oxide (10 mg /L) was added after medium sterilization and before inoculation. The flasks were incubated at the same above conditions for 72 h and selenium uptake was determined for each mutant and the original strain.

II-High capacity of selenium uptake by superior mutants

Selenium (IV) oxide (100 mg /L) was added after the original strain and superior mutants were gown at the same above conditions for 48 h. The flasks were incubated again under the same conditions for 24 h. Selenium uptake was determined for each superior mutant and the original strain.

E- Protoplast formation from *S. cerevisiae*

Yeast cells were grown in 50 ml of protoplasting medium and incubated on a rotary shaking incubator (150 rpm) at 30^{o}C for 18 hours. Cells were collected by centrifugation and washed twice with sterile distilled water. Washed cells were resuspended in the pre-treatment solution, then the suspension was incubated for 20 min at 35^{o}C with gentile agitation. After incubation, cells were centrifuged again and resuspended in a protoplasting buffer containing snail enzyme (1%, w/v) and incubated under shaking (120 rpm) in a water bath at 35^{o}C. Cells were checked periodically, using phase- contrast microscope for the formation of protoplasts. The conversion of cells into protoplasts was completed within one hour of incubation. Protoplasts were centrifuged at 2500 rpm for 5 min at 4^{o}C and resuspended in protoplasting buffer.

F- Porotoplast fusion

Protoplast fusion could be carried out between selected isolates which differ in either their nutrient requirements; resistance to antibiotics or selenium uptake levels as follow : Protoplasts from different cultures were mixed, centrifuged at 2500 rpm for 5 min and the supernatant was removed. Two ml of PEG solution were added and mixed gently with protoplasts. The mixture of protoplasts was incubated up to 40-60 min at 30^{o}C . Then, the mixture was diluted with 0.65 M KCl. Samples of 0.1 and 0.5 ml of the dilutions were overlayed on the regeneration medium.

G- Isolation of total DNA from yeast strains

Total DNA was isolated according to Khattab (1997). The quantity and purity of the obtained DNA were determined according to the UV-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240) according to Sambrook *et al.* (1989).

H- Molecular analysis polymerase chain reaction (PCR)

For PCR technique, Ready-To-Go PCR Beads (Amersham, Pharmacia Biotech. No. 27-9555-01) were used. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Table (1) presents the three different primers which were used in the present study. The first and third primers supplied by Metabion International AG, Germany. The second one supplied by Operon Technologies Company, Netherlands. To each Ready To Go PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water.

Table (1): The nucleotides sequences of the applied primers.

Serial No.	Type of primer	Code No.	Nucleotides sequence
1	Random	opb-15	5'- GACGGATCAG -3'
2	Random	opa-2	5'- TGCCGAGCTG -3'
3	Random	opb-08	5'- GTCCACACGG -3'

The amplification protocol was carried out as follows:

1. Denaturation at 95^{o}C for five min.

2. Thirty-five cycles each consists of the following segments: a. Denaturation at 95^{o}C for one min; b. Primer annealing at 37^{o}C for two min. according to

GC ratio of each primer; c. Incubation at 72°C for two min. for DNA polymerization.
3. Hold at 4°C till analysis. Finally, the amplified DNA products from RAPD analysis were electrophorated on 1.5% agarose gel and 1 X TBE buffer at consistent 100 volt for about 2 hrs. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV Transeleminator.

I- Determination of selenium uptake

Revanasiddappa and kumar (2001) described a rapid and sensitive spectrophotometric method for the determination of trace amounts of selenium using Variamine Blue (VB) as a chromogenic reagent. An aliquot of a sample solution containing 2 to 20 µg of selenium was transferred into a series of 10 ml calibrated flasks. Volumes of 1 ml of 2% potassium iodide, followed by 1ml of 2 M HCl, were added to it. The mixture was gently shaken until the appearance of yellow color, indicating the liberation of iodine. Then , 0.5 ml of 0.05 % Variamine Blue (VB) was added to it , followed by the addition of 2 ml of a 1 M sodium acetate solution . The contents were diluted to the mark with distilled water and mixed well. The absorbance of the colored samples was measured at 546 nm against the corresponding reagent blank. Shimadzu UV–VIS spectrophotometer model UV-240 with 1 cm matched quartz cells were used for all absorbance measurements.

3.Results and Discussion

Effect of high selenium concentration on yeast growth

Influence of different selenium (IV) oxide concentrations on yeast growth of several growing yeast cultures was shown in Table 1. Results showed negative correlation between selenium concentration and growth yield for all tested strains. *C. utilis* showed sensitivity to selenium oxide since the percentage of decreasing growth was 34.04 & 21.70 at selenium conc. of 5 & 10 mg/l, respectively. Selenite [Se (IV)] is assimilated by microorganisms and reduced to Se (Π) which is covalent bound to C as selenomethionine. Further, a common metal-induced response in yeast is intracellular synthesis of cysteine-rich metal-binding proteins called methallothioneins which have functions in metal detoxification and also storage and regulation of the intracellular metal ion concentrations (Gadd, 1986). *S. cerevisiae* which exhibited the greatest tolerance to all selenium concentrations was selected and subjected to mutagenic treatment with different doses of ultraviolet light.

Evaluation of uptake of selenium (IV) oxide by yeast cells

Data in Table (2) showed that all yeast strains exhibited high selenium uptake at concentration of 5 mg/l. It was 55, 47.6 and 45 % for *S. cerevisiae, C. utilis* and *K. marximanus*, respectively. At the two level of selenium concentrations (5 and 10 mg/l), *S. cerevisiae* exhibited the high selenium uptake and accumulation. Cobo-Fernandz *et al*. (1995) suggested that Se (IV) uptake occurs along with conversion of Se (IV) into other selenium species, probably Se (Π) as selenomethionine or selenocysteine, which do not generate hydrogen selenite under the same experimental conditions as Se (IV) does.

UV-Mutagenesis and selenium resistant mutants

Data presented in Table (3) showed selenium resistant mutants obtained after ultraviolet (UV) mutagenesis of *S. cerevisiae*. Results showed that the highest percentage of resistant mutants (29.63) was obtained after 20 min of UV treatment whereas the lowest percentage (4.71) was obtained after one min. exposure time. On the other hand, 5 and 10 min UV-treatments gave nearly the same percentages (15& 15.9, respectively) of resistant mutants. Neither 30 min nor zero min UV treatments produced selenium resistant mutants.

Evaluation of mutants for selenium uptake

The uptake of selenium by yeast cells was studied from 72 h samples taken from each flask. Yeast cells were centrifuged and washed three times with 1 ml of water. The incorporation of selenium into yeast cells are presented in Table (4). The results in this table showed that there occurs a clear stimulation of the selenium uptake by yeast cells after UV-irradiation.

Selenium uptake of all mutants, grown in the presence of selenium oxide (10 mg/L), was higher than the original strain which incorporate 39.76 (mg/L) of selenium. Increasing selenium uptake is ranged from 178.78 to 408.16% in comparison with the original strain (100%). Five mutants, Nos. 5-8, 15-5, 20-1, 25-1 and 25-2 uptake nearly all selenium present in growth medium which represents more than four fold selenium uptake by the original strain.

High capacity of selenium uptake by superior mutants

Table (5) shows selenium uptake of superior yeast mutants in the presence of selenium oxide (100 mg/L). Results showed that yeast cells tolerate high selenium concentration in the growing medium and uptake percentages ranging from 46.84 to 79.38. The superior mutant No. 5-8 uptake 79.38 mg/L selenium

which represents 199.65% of the original strain. However, all mutants exhibited more selenium uptake than the original strain except for mutant No. 5-5 which exhibited slight increase (17.81%) in comparison with the original strain. Nine mutants, Nos. 5-3, 5-8, 5-9, 15-5 , 15-6 , 20-1 , 20-8 and 25-1 which represents about 81.82% of the mutants, uptake more than 50% of selenium than the original strain. Our results with mutation are consistent with those of Ramsay and Gadd (1997). They observed many differences between mutants and wild type of *S. cerevisiae* to several toxic metals. Moreover, Gharieb and Gadd (1998 and 2004) found many differences between vacuolar – lacking strains and the defective mutants of S. *cerevisiae* to uptake of selenium. On the other hand, our results are not agreement with those reported by Caker *et al.* (2009). They indicated that the EMS- treatment didn't significantly increase the sensitivity or resistance of yeast cells to cobalt ions.

Response of superior mutants to different antifungal

In order to investigate the effect of protoplast fusion on selenium uptake, six induced mutants which exhibited the highest selenium uptake were selected to be used as parental isolates for protoplast fusion crosses. Moreover, an additional marker, i.e. antifungal resistance or sensitivity were determined for the selected mutants.

Data presented in Table (6) showed the resistance or sensitivity to benomyl, cycloheximide, Griseofulvin, nystatin and miconazole. Results showed that, although the original strain was resistance to benomyl, cycloheximide and miconazole and sensitive to the other antifungal (griseofulvin and nystatin), all the selected mutants exhibited different antifungal response to all of the tested antifungal. Mutant 25/1 proved to be resistant to all of the tested antifungal except griseofulvin. Three mutants (5/8, 5/9 and15/6) were resistant to two antifungal, mutants 5/8 and 15/16 were resistant to cycloheximide while mutants 5/9 and 15/6 were resistance to miconazole. One mutant (5/3) exhibited resistance to three and sensitive to two antifungal. The last mutant, i.e., 25/1 showed the highest level of antifungal resistance, since it resistant to four antifungal.

However, three mutants (5/8, 5/9 and 20/1) were selected and introduced subsequently into protoplasting, fusion and regeneration. Two mutants, i.e., 5/8 and 5/9 were used as parental strains for cross 1 where benomyl and cyclohexamide or nystatin and miconazole was used as selective markers. On the other hand, 5/8 and 20/1 were used for cross 2 where griseofulvin and cycloheximide or griseofulvin and nystatin was used as selective markers.

Protoplast Fusion

Among the wide variety of strain improvement techniques, protoplast fusion seems to be an efficient way to induce genetic recombination and polyplodization in yeast (Gupthar and Garnett 1987and Gupthar, 1992). This method has also been proved to be valuable in the development of new industrial yeast strain. However, under the conditions mentioned under materials and methods, protoplasts can be isolated as shown in Fig(1/B).The increase of volume in comparison with the intact cells Fig.(1/A) was apparently due to osmotic enlargement of the vacuole(Svoboda,1978).

On the other hand, the addition of polyethylene glycol to the protoplast suspension resulted in intensive agglutination which leads to the formation of large aggregates which is a precondition for fusion. Furthermore, the presence of calcium ions is normally an important requirement for fusion of the aggregated protoplasts (Peberdy and Ferenczy, 1985). Any how, when the PEG-treated protoplast suspension was embedded into solid regeneration medium, some of them increased in volume and reverted to normal cells. Cell clones with normal cell morphology and prototrophic properties were selected after twice-repeated single-colony isolation and storage on slant agar medium at 4^0C for further genetic analysis. Fusants obtained will evaluate for their tolerance, uptake and accumulation of selenium in comparison with the original strain.

Evaluation of fusants for selenium uptake

Data in Tables 7 and 8 presents the obtained fusants from the crosses which were carried between different mutants. The results obtained showed that 38 fusants were obtained from the cross No. 1 and 36 fusants from the cross No. 2.

Results in Table (7) revealed that all obtained fusants exhibited higher selenium uptake than the original strain (*S. cerevisiae* NRRL Y-139). Moreover, four fusants (obtained from cross1 selected under miconazole and nystatin as selective markers and designated MN) exhibited higher selenium uptake of about 219% in comparison of the original strain. Selenium uptake of these fusants; designated MN2, MN9, MN12 and MN13, represents about 110% and 117% of parent 1 and parent 2, respectively. It's interesting to note that all fusants exhibited higher selenium uptake than its parent 2 (5/9).Only one fusants, i.e. MN11 showed a slight increasing of 1.24% of selenium uptake than parent 2 (5/9). On the other hand, fusants obtained also from cross 1 between (5/8 and 5/9 mutants) in the presence of benomyle and cycloheximide as selective markers. Data showed that 11 fusants out of 18 (designated BC)

uptake selenium more than its higher parental strain (5/8). Their selenium uptake were ranging from 80.29 to 84.58 mg/L which represents about 101 to 107%, 108 to 114% and 202 to 213% in comparison with parent 1, parent 2 and wild type, respectively. In addition, only one fusant, i.e. BC15 failed to reach the level of selenium uptake of both parents (5/8 and 5/9). However, BC12 and BC13 were considered as promising fusants which uptake more than two fold of the original strain.

Moreover, the effect of intra-specific protoplast fusion technique on selenium uptake were evaluated on 36 fusants selected from cross 2 between 5/8 and 20/1 mutants in the presence of cycloheximide, griseofulvin and nystatin. Data presented in Table (8) showed that out of 20 fusants, obtained in the presence of griseofulvin and nystatin as selective markers, ten fusants designated GN exhibited more selenium uptake than its higher parental mutant (5/8). Their selenium uptake percentages to wild type strain ranged from nearly 202 to 211 which represent about 101% to 106 % in comparison with the higher parent. Only three fusants, i.e. GN7, GN9 and GN 17 with higher selenium uptake than their higher parent strains (5/8 and 20/1), were obtained from cross 2.

On the other hand, 16 fusants obtained from cross 2 in the presence of cycloheximide and griseofulvin designated CG. Results in Table (8) showed that 62.5% of the fusants (10 fusants) exhibited more selenium uptake than its higher parent (5/8). Increasing selenium uptake is ranging from 79.60 to 82.57 which representing (100.28 to 104.02, 110.16 to 114.27) and (200.20 to 207.67) percentages in comparison with parent 1, parent 2 and the original strain, respectively. However, fusants selected from cross 2 in the presence of cycloheximide, griseofulvin and nystatin as selective markers proved to be less effective for increasing selenium uptake than fusants obtained from cross 1 in the presence of miconazoale, nystatin, benomyl and cycloheximide. In general, it could be concluded that ultraviolet mutation proved to be an effective technique to enhance selenium uptake. In addition, intra-specific protoplast fusion between higher selenium uptake mutants proved to be effective to achieve superior selenium uptake fusants. Therefore, protoplast fusion have been used successfully to enhancement the selenium uptake. The recombinants strains can be obtained by this technique (Lin *et al.*, 2005 ; Abdel Salam and Khattab 2005).

Random amplified polymorphic DNA (RAPD) analysis:

An attempt was conducted to evaluate the genetic effect of mutagenic treatment and protoplast fusion on the DNA nucleotide sequence of the mutants and protoplast fusants compared to the original strain (*S. cerevisiae* NRRL-Y-139).

Using primer No.1 (Figure 2), it was clearly noticed that six amplified bands were occurred when DNA of the original strain was used as a template. The bands sizes were 2100, 900, 650, 500,380 and 200 bp as shown in lane. Furthermore, the first band (2100bp was disappeared with the mutant No.20-1, fusants Nos. BC13 and MN (lanes 5, 6 and 7) and the second band (900bp) did not detected with the fusant BC13 (lane 6).

On the other hand, the three superior fusants No., BC13, MN9 and MN13 appeared a new amplified bands with the size of 1500 bp (lanes 6, 7 and 8) which did not detected with the original and parental strains. In addition, two new amplified bands with the size of 1250 and 1000 bp were detected with two superior fusants Nos. CG16 and GN17 (lanes 9 and 10). Furthermore, when DNA samples of the superior fusants Nos. MN9 and MN13 were used as templates with primer Nos. a new amplified band with size of 800 bp was detected. Finally, using primer No.1 with DNA templates of the fusants No., CG16 and GN17, a new amplified band with size of 250 bp was detected.

The application of primer No. 2 (Figure, 3) against strain used in RAPD analysis induced only one monomorphic amplified band with size of 2100bp . It was interested to notice that mutants Nos. 5-8 and 5-9 (lanes 3 and 4) which have about 99 % selenium uptake more than the original strain, exhibited high intensity band. The same finding was detected with the superior fusant No. CG16 (lane 9). In general all mutants and fusants exhibited high intensity band compared to the original strain.

Regarding the application of the primer No. 3 (Figure, 4), it was clearly noticed that most of the tested mutants and fusants (lanes 2-5 and 8, 9) proved to have three distinct amplified bands which identical of its original strain. With two exceptions of the fusants Nos. CG16 and GN17 (lanes 9 and 10) exhibited new amplified band with size of 500 bp. Moreover, two fusants Nos. MN9 and MN13 (lanes 7 and 8) present different banding pattern, since two distinct bands with different size were detected (700 and 1100 bp).

The above differences in RAPD profiles confirmed the evidence of genetic variations in yeast genome after the UV- mutagensis and protoplast fusion technique. Furthermore, some of these differences based on RAPD technique, could be used as genetic markers for genetic diversity of selenium uptake characteristics.

Table (1): Effect of different selenium (IV) oxide concentrations on yeast growth* ability after 72 hours incubation at 30º C .

Yeast strains	Selenium conc. (mg/L)			
	0	2	5	10
S. cerevisiae	1.004	0.846	0.713	0.526
C. utilis	1.93	1.72	0.664	0.419
K. marxianus	1.36	1.154	0.993	0.414

* Measured as optical density at 600 nm.

Table (2): Evaluation of selenium (IV) oxide uptake by different yeast cells after 72 incubation at 30º C

Yeast strain	selenium conc. (mg/L)			% Uptake
	Initial	Residual	Uptake	
S. cerevisiae	5	2.25	2.75	55
S. cerevisiae	10	7.55	2.45	24.5
C .utilis	5	2.62	2.38	47.6
C .utilis	10	7.95	2.05	20.5
K. marxianus	5	2.75	2.25	45
K. marxianus	10	9.95	0.05	0.5

Table(3):Selenium resistant mutants obtained after ultraviolet (UV) mutagenesis

Exposure time(min)	No. of tested colonies	No. of resistant colonies*	% of resistant colonies
0	192	0	0
1	85	4	4.71
5	60	9	15.00
10	44	7	15.91
15	35	8	22.86
20	27	8	29.63
25	15	3	20.00
30	8	0	0

*Mutants were screened on YEPD agar medium containing selenium oxide (20 mg /L).

Table (4): Selenium uptake of selected selenium resistant mutants grown in the presence of selenium oxide (10 mg /L).

Mutant No.	Se.*** uptake(mg/L)	% to W.T.	Mutant No.	Se. uptake (mg/L)	% to W.T.
W.T.	2.45	100.00	10-7	5.90	240.82
1*-1**	6.37	260.00	15-1	7.35	300.00
1-2	5.5	224.49	15-2	6.81	277.96
1-3	9.46	386.12	15-3	5.50	224.49
1-4	6.47	264.08	15-4	8.00	326.53
5-1	5.80	236.73	15-5	10.00	408.16
5-2	8.72	355.92	15-6	9.50	387.76
5-3	9.01	367.76	15-7	8.17	333.47
5-4	6.56	267.76	15-8	7.80	318.37
5-5	9.24	377.14	20-1	10.00	408.16

5-6	4.38	178.78	20-2	7.70	314.29
5-7	8.81	359.59	20-3	7.46	304.49
5-8	10.00	408.16	20-4	6.00	244.90
5-9	9.87	402.86	20-5	8.39	342.45
10-1	7.65	312.24	20-6	7.52	306.94
10-2	7.91	322.86	20-7	5.09	207.76
10-3	6.36	259.59	20-8	9.72	396.73
10-4	8.40	342.86	25-1	10.00	408.16
10-5	7.82	319.18	25-2	10.00	408.16
10-6	8.34	340.41	25-3	8.17	333.47

***Time of treatment (min)**

****Mutant No.**

***** Seleniu**

Table (5): Selenium uptake of superior selenium resistant mutants grown in the presence of selenium oxide (100 mg/L).

Mutant No.	Residual Se. (mg /L)	Se. uptake	
		uptake (mg/L)	% to W.T.
W.T.	60.24	39.76	100.00
1-3	45.72	54.28	136.52
5-3	34.80	65.20	163.98
5-5	53.16	46.84	117.81
5-8	20.62	79.38	199.65
5-9	25.74	74.26	186.77
15-5	39.52	60.48	152.11
15-6	28.59	71.41	179.60
20-1	27.74	72.26	181.74
20-8	37.38	62.62	157.49
25-1	36.75	63.25	159.08
25-2	41.65	58.35	146.76

Table (6): Antifungal response of the original strain (W.T) and the superior mutants selected for protoplast fusion

Mutant No.	Antifungal response				
	B	C	G	N	M
W.T	+	+	-	-	+
5-3	+	-	+	-	+
5-8	-	+	-	+	-
5-9	+	-	-	-	+
15-6	-	+	-	-	+
20-1	-	-	+	-	-
25-1	+	+	-	+	+

B**:** Benomyl (5 µg/ml), **C:** Cycloheximide (100 µg/ml), **G:** Griseofulvin (250 µg/ml) , **N:** Nystatin 10 µg/ml) , **M:** Miconazole (5 µg/ml)

(+) : Resistant , **(-):** Sensitive

Table (7): Selenium (Se) uptake of fusants obtained after protoplast fusion between 5/8 and 5/9 mutants (cross 1) .

Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.	Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.
W.T	60.24	39.76	50.09	53.54	100	W.T	60.24	39.76	50.09	53.54	100
P_1 5/8	20.62	79.38	100	106.89	199.65	P1 5/8	20.62	79.38	100	106.89	199.65
P_2 5/9	25.74	74.26	93.55	100	186.77	P2 5/9	25.74	74.26	93.55	100	186.77
MN 1*	18.75	81.25	102.36	109.41	204.35	BC1**	17.30	82.70	104.18	111.37	208.00
MN 2	12.90	87.10	109.73	117.29	219.06	BC 2	19.52	80.48	101.39	108.38	202.41
MN 3	15.23	84.77	106.79	114.15	213.20	BC 3	22.48	77.52	97.66	104.39	194.97
MN 4	14.61	85.39	107.57	114.99	214.76	BC 4	20.65	79.35	99.96	106.85	199.57
MN 5	17.74	82.26	103.63	110.77	206.89	BC 5	17.70	82.30	103.68	110.83	206.99
MN 6	14.28	85.72	107.99	115.43	215.59	BC 6	24.37	75.63	95.28	101.84	190.22
MN 7	22.37	77.63	97.80	104.54	195.25	BC 7	30.29	69.71	87.82	93.87	175.33
MN 8	19.08	80.92	101.94	108.97	203.52	BC 8	25.06	74.94	94.41	100.92	188.48
MN 9	12.80	87.20	109.85	117.43	219.32	BC 9	19.71	80.29	101.15	108.12	201.94
MN 10	21.76	78.24	98.56	105.36	196.78	BC 10	18.88	81.12	102.20	109.24	204.02
MN 11	24.50	75.50	95.11	101.67	189.89	BC 11	18.54	81.46	102.62	109.70	204.88
MN 12	13.19	86.81	109.36	116.90	218.34	BC 12	15.76	84.24	106.12	113.44	211.87
MN 13	12.68	87.32	110.00	117.59	219.62	BC 13	15.42	84.58	106.55	113.90	212.7
MN 14	14.94	85.06	107.16	114.54	213.93	BC 14	17.27	82.73	104.22	111.41	208.07
MN 15	23.45	76.55	96.44	103.08	192.53	BC 15	27.31	72.69	91.57	97.89	182.82
MN 16	20.21	79.79	100.52	107.45	200.68	BC 16	18.70	81.30	102.42	109.48	204.48
MN 17	19.42	80.58	101.51	108.51	202.66	BC 17	17.13	82.87	104.40	111.59	208.43
MN 18	16.55	83.45	105.13	112.38	209.88	BC 18	23.95	76.05	95.81	102.41	191.27
MN 19	18.36	81.64	102.85	109.94	205.33						
MN 20	17.40	82.60	104.06	111.23	207.75						

*Fusants obtained under miconazole and nystatin as selective markers

**Fusants obtained under benomyl and cycloheximide as selective markers

Table (8): Selenium (Se) uptake of fusants obtained after protoplast fusion between 5/8 and 20/1 mutants (cross 2).

Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.	Fusant No.	Se residual (mg/L)	Se uptake (mg/L)	% to P_1	% to P_2	% to W.T.
W.T	60.24	39.76	50.09	53.54	100	W.T	60.24	39.76	50.09	53.54	100

P_1 5/8	20.62	79.38	100	106.89	199.65	P_1 5/8	20.62	79.38	100	106.89	199.65
P_2 20/1	27.74	72.26	91.03	100	181.74	P_2 20/1	27.74	72.26	91.03	100	181.74
GN 1*	24.89	75.11	94.62	103.94	188.91	CG 1**	21.70	78.30	98.64	108.36	196.93
GN 2	19.55	80.45	101.35	111.33	202.34	CG 2	18.32	81.68	102.90	113.04	205.43
GN 3	20.71	79.29	99.89	109.73	199.42	CG 3	23.62	76.38	96.22	105.70	192.10
GN 4	17.37	82.63	104.09	114.35	207.82	CG 4	25.38	74.62	94.01	103.27	187.68
GN 5	22.60	77.40	97.51	107.11	194.67	CG 5	19.92	80.08	100.88	110.82	201.41
GN 6	18.83	81.17	102.25	112.33	204.15	CG 6	23.65	76.35	96.18	105.66	192.03
GN 7	16.18	83.82	105.60	115.99	210.81	CG 7	17.81	82.19	103.54	113.74	206.72
GN 8	22.46	77.54	97.68	107.31	195.02	CG 8	20.40	79.60	100.28	110.16	200.20
GN 9	16.25	83.75	105.51	115.90	210.64	CG 9	24.50	75.50	95.11	104.48	189.89
GN 10	23.90	76.10	95.87	105.31	191.40	CG 10	23.47	76.53	96.41	105.91	192.48
GN 11	22.32	77.68	97.86	107.50	195.37	CG 11	20.27	79.73	100.44	110.34	200.53
GN 12	17.35	82.65	104.12	114.38	207.87	CG 12	17.86	82.14	103.48	113.67	206.59
GN 13	19.70	80.30	101.16	111.13	201.96	CG 13	19.45	80.55	101.47	111.47	202.59
GN 14	20.42	79.58	100.25	110.13	200.15	CG 14	18.29	81.71	102.94	113.07	205.51
GN 15	18.66	81.34	102.47	112.57	204.58	CG 15	20.16	79.84	100.58	110.49	200.80
GN 16	20.48	79.54	100.20	110.07	200.00	CG 16	17.43	82.57	104.02	114.27	207.67
GN 17	16.20	83.80	105.56	115.97	210.76						
GN 18	21.52	78.48	98.866	108.61	197.38						
GN 19	17.97	82.03	103.34	113.52	206.31						
GN 20	20.51	79.49	100.14	110.01	199.92						

*Fusants obtained under griseofulvin and nystatin as selective markers

**Fusants obtained under cycloheximide and griseofulvin as selective markers

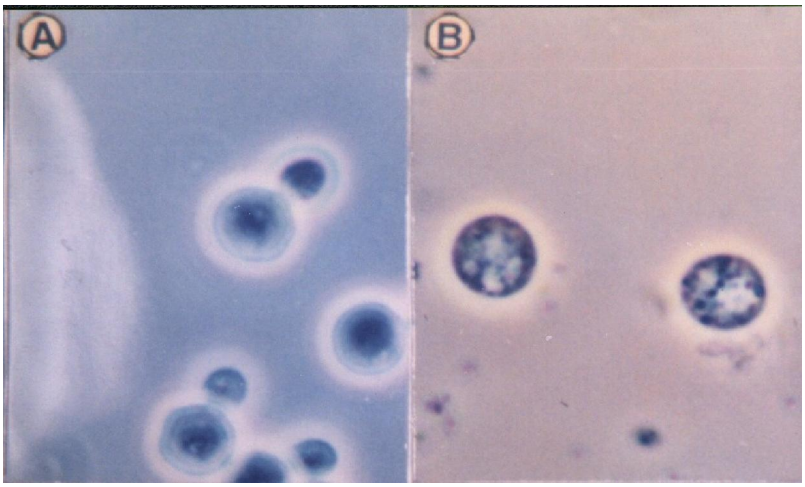

Figure (1): Micrographs represent formation of yeast protoplasts (B) in comparison with the original parent (A).

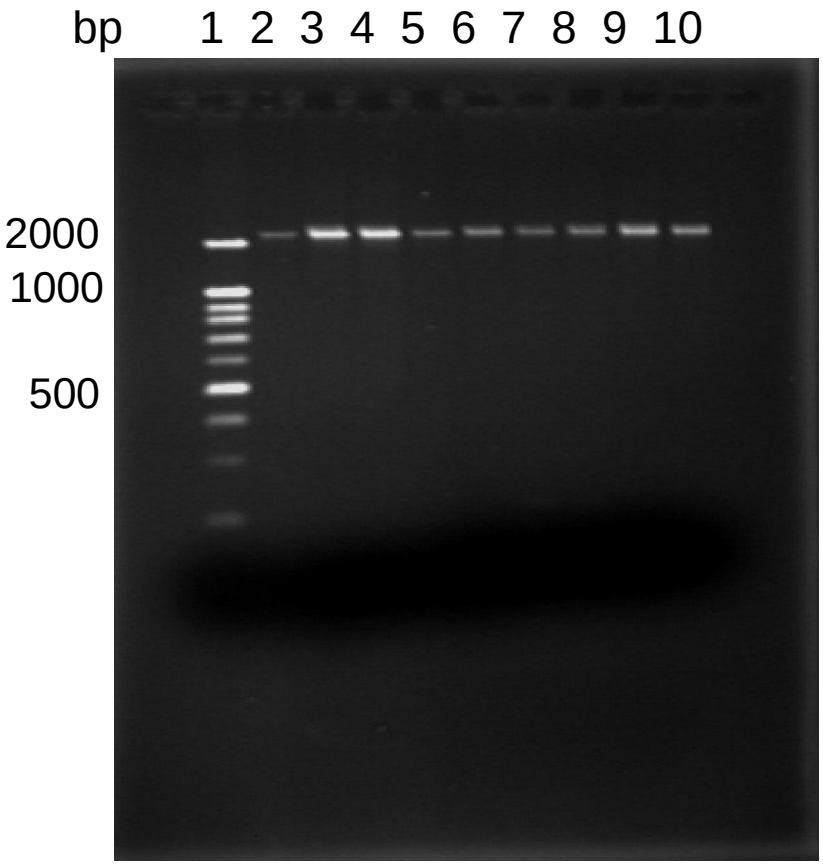

Figure (2): Photographs of DNA amplified banding patterns based on RAPD for three parental mutants and five protoplast fusants against original strain (lane 2) and 100 bp loader DNA marker (lane 1) using primer No.1. Mutant sequence as follows: (lane 3 to 5), 5-8.5-9 and 20-1.Fusant sequences (lanes 6 to 10), BC13, MN9, MN13, CG16 and GN17.

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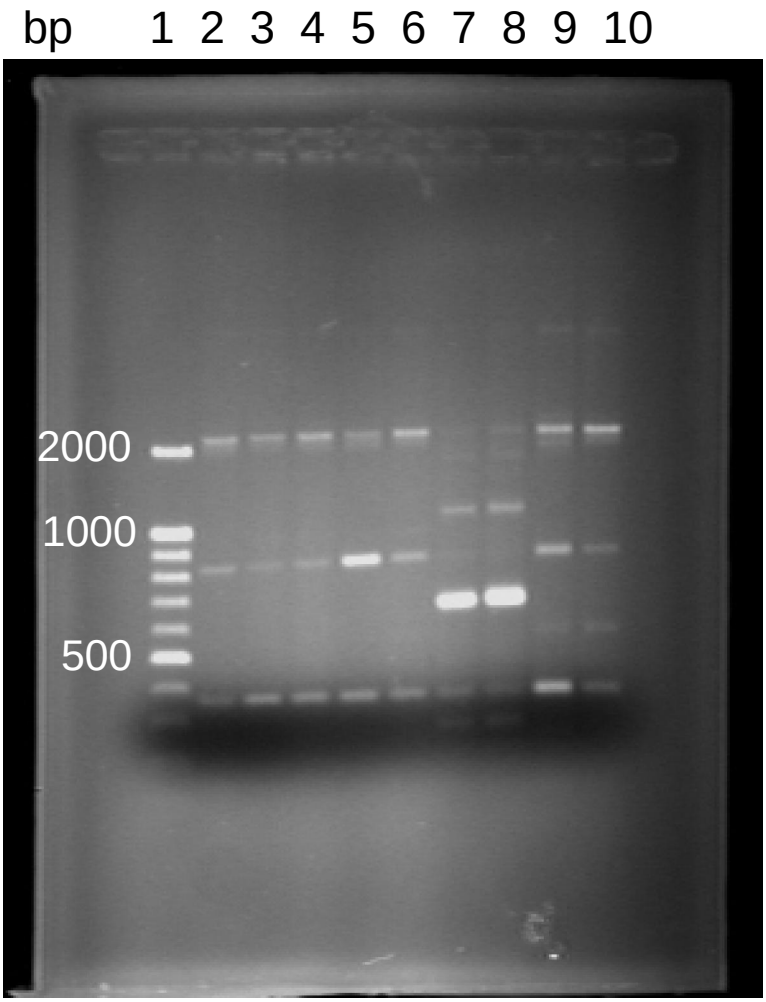

Figure (3): Photographs of DNA amplified banding patterns based on RAPD for three parental mutants and five protoplast fusants against original strain (lane 2) and 100 bp ladder DNA marker (lane 1) using primer No.3. Mutant sequences as follows: (lane 3 to 5), 5-8, 5-9 and 20-1. Fusants sequence (lanes 6 to 10), BC13, MN9, MN13, CG16 and GN17.

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