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RAPD Analysis of DNA Polymorphism in the Yeast *Hansenula anomala* and *Rhodotorula rubra* Irradiated by Gamma and Fast Neutrons

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ABSTRACT

The influence of gamma and fast neutrons irradiations on cell killing and DNA polymorphism in two fungal yeasts, *Hansenula anomala* and *Rhodotorula rubra* has been studied. 3 x 10^5 RAD gamma rays resulted in killing 38.0 and 22.7% of the two fungal yeast cells, respectively, whereas 3 RAD of fast neutrons caused killing of 45.7 and 49.8%, respectively. The polymerase chain reaction (PCR) that includes random amplified polymorphic DNA (RAPD) was employed to investigate the influence of ionizing radiation in inducing DNA-Polymorphisms. Number of amplified DNA fragments ranged between 81 and 78 in *R. rubra and H. anomala* respectively, when 3 M RAD of gamma rays were used. It seems that fast neutrons were more efficient in inducing DNA polymorphism than gamma rays.

Key Words: RAPD; Analysis*; Hansenula anomala; Rhodotorula rubra;* Gamma and neutrons irradiation

INTRODUCTION

H. anomala has normal ploidy as diploid vegetative cells with hat shaped, ascospores, and is strongly fermentative, usually forming mats, or often folded pellicles, which produce esters with a strong odor of ethyl acetate and polymers containing phosphorous (i.e. it produce extracellular phosphomannans). *H. anomala* is common in soil. The mat form, characteristically produces more lipids and less polysaccharide inside and on the cell wall. It is the best example of yeasts, which produce both the highly oxidative and the fermentative colony forms which can efficiently assimilate or oxidize alcohols and organic acids (Sydow & Sydow, 1919). Harrison (1928) established the genus *Rhodotorula* for the asporogenous and pigment-forming yeasts. (Spencer *et al*., 1964) to emphasized the ability to synthesize starch as one of the primary criteria for generic differentiation as pointed before by (Wickerham, 1952). The species remaining in *Rhodotorula* are those in which the capsular polysaccharide consists of a mannan (Gorin *et al*., 1965) with alternating linkages β1 → 3 and β1 → 4 bonds. A sexual stage in the genus *Rhodotorula* was reported for the first time by Banno (1963). *R. rubra* is an extremely common species and world wide in distribution terrestrial as well as marine. It is one of the most common *Rhodotorula* species found in clinical specimens especially the gastro intestinal tract of man, these strains all grow at 37ºC and are resistant to cycloheximide. *R. rubra* appears to be a stable physiological species; although morphological changes have been noted, such as mutations from smooth to wrinkled colonies (Lodder & Kreger Van. Ru, 1952). LET (Linear Energy Transfer) radiation such as gamma rays characteristically produce a different pattern and distribution of DNA fragmentation than those of high-LET radiation such as fast neutrons in both prokaryotic and eukaryotic organisms (Goodhead & Nikjoo, 1989; Prise *et al*., 1989). It is believed that low LET radiation produces a higher ratio of DNA SSBS (single-stranded breaks) to DSBS (double-stranded breaks), per unit dose than high-LET radiation (peak *et al*., 1988; Fox & Mcnally, 1999). DNA lesions may be recognized and repaired by more than one DNA-repair processes. The induction of error-free recombinational repair and error-prone repair were observed upon exposure yeast cells to combinations of mutagens. Results indicated that lesions were recognized by both recombinational repair systems (Mitchel & Morrison, 1987; Boreham & Mitchel, 1991). However DNA, SSB may be an important lesion that signals the induction of the recombinational repair mechanism (Naresh & Rakesh, 2001). Polymerase chain reaction (PCR), which include random amplified polymorphic DNA (RAPD) is a useful analysis tool for detecting the polymorphism in DNA sequence (Williams *et al*., 1990; Powell *et al*., 1996; Cao *et al*., 1999; Garcia *et al*., 2000; Roldan *et al*., 2000). The advantage of RAPD over other molecular markers is the low technical input and small quantity of DNA needed for the analysis (Hernandez *et al*., 1999; Manabe *et al*., 1999). The aim of this study was to investigate DNA polymorphism generated by gamma rays and fast neutrons in two fungal yeasts that were isolated from Zagazig city, Egypt, *Hansenula anomala* and *Rhodotorula rubra*. Two different aspects were studied, resistance to cell killing and DNA fragmentation.

MATERIALS AND METHODS

Fungal yeast species. Two ascosporogenous fungal yeasts, *Hansenula anomala*, and *Rhodotorula rubra* were isolated from Zagazig airospora (El-Sherbeny, 1999), and they were identified according to (Lodder, 1970) these isolates were used in this study, Sub-culturing for routine work was carried on Sabouraud's dextrose agar medium (pH 4.6, incubation temp. at 30ºC).

Growth conditions. Yeast cells were inoculated at 23ºC in 200 mL of nutrient broth (YES) media containing 0.1% yeast extract, 170 g yeast nitrogen base with amino acids, 1% Sodium succinate and 2% glucose prepared in baffled flasks. The cultures were grown on a shaker at 300 rpm for 3 days at 30ºC. The optical density of the cells has been measured at 550 nm. Viable counts were assayed on malt extract medium using coulter counter, and cfu/mL has been calculated.

Gamma rays irradiation. The gamma chamber 400 A (Isotope Group, India) of the Radiation Research and Technology Center, Nasr city, Cairo, Egypt was used to irradiate gamma rays. The gamma irradiation doses were performed from ^{60}Co source, which was monthly estimated with a rate of 0.033 k Gy/ min. Samples were irradiated for different time periods to reach the demanded doses. Ten millimeter of cell suspension were exposed to three doses of radiation, (1, 2, & 3 M RAD), and survival percentages were determined by plating the cell suspension on YEPS (0.1% Yeast extract, 1% Yeast nitrogen base with amino acids 1% sodium succinate, 2% peptone and 2% glucose).

Fast neutrons irradiation. Ten ml of cell suspension were irradiated with fission neutrons from Cf 252 point (Radiochemical center, Amercham, England) at, Biophysics Department Faculty Science, Cairo University. Cf 252 source has a very small volume (50 μg), and we considered it as a point source. The neutron flux (ϕ) at a distance (r) from the point source, which emits neutrons at the rate (q) per second after irradiation time (t) is equal to $\varphi = \frac{qt}{4\pi r^2}$ n/m^2 or n/ cm^2. The activity of the source at the time of irradiation was calculated from the usual formula $N = No\ e^{-\lambda t}$. Our neutron source emits 8 x 10^5 n/sec at the beginning of the experiment. We put the samples at different distance from the source and for different times to reach the demanded doses. Three doses (0.03, 0.3, 3 RAD) have been used.

DNA isolation. Cultures of yeast derived from cells surviving ionizing radiation were grown on Sabouraud agar plates for 24 h at 30ºC. Single colonies were picked up and inoculated into 200 mL of YEPD broth medium (2 g yeast extract, 2 g peptone, 2 g glucose) then incubated at 30ºC for 36 h. Genomic DNA was isolated according to (Vazquez *et al*., 1991) and purified according to Promega corporation procedure (1999) for the RAPD analysis.

RAPD analysis. The ten 10-mer random primers used in this study were selected from the Operon Kits (Operon technologies Inc. Alameda CA) and are listed in Table III.

RAPD analysis. PCR reactions were carried out in a volume of 50 μL containing 5 μL of 10 x buffer, 50 mM of $MgCl_2$, 1 μL of 2 mM dNTPS, 1 μL of 10 μ M of each primer, 1 μL of 5U/mL tag-polymerase, 1 μL of 10 ng/U template DNA and 41 μL of sterile double distilled water.

DNA amplification was carried out in a Perkin Elmer Gene AMP system 2400 thermocyle programmed for 47 cycles. Amplified samples were analyzed by electrophoresis in 1.4% agarose gels. Each lane contained different samples as follows: 1 = MDNA molecular weight standard, 2 = un-irradiated *R. rubra*; 3 = irradiated *R. rubra* (3 RAD, fast neutrons); 4 = *R. rubra* irradiated (3 M RAD, gamma rays); 5 = un-irradiated *H. anomala*; 6 = irradiated *H. anomala* (3 RAD, fast neutrons); 7 = irradiated *H. anomola* (3 M RAD, gamma rays). The RAPD patterns were visualized with a UV transilluminator and photographed using Polaroid Camera (MP_4 land Camera.).

Statistical analysis. The data were statistically analyzed as a complete randomized block design. The means was compared by analysis of variance (ANOVA) (Hicks, 1983).

RESULTS AND DISCUSSION

Radiation induced radio resistance. The effect of various doses of gamma rays (1-3 M RAD) on cell survival of *H. anomala* and *R. rubra is shown* in (Table I). Survival percentages of the two fungal yeasts reached up to 62 - 77.3 upon exposure to 3 M RAD, respectively. The killing percentages ranged up from 22.7 up to 38.0. On the other hand, the survival percentages upon exposure to fast neutrons were dramatically declined (Table II). and the killing percentages recorded were 45.7 up to 49.8 when 3 RAD was used. It seems that both fungal yeasts employed in this investigation exhibited resistance upon exposure to ionizing radiation. Therefore, irradiation with low doses of ionizing radiation may enhance the capability for recombinational repair systems in *H-anomala* and *R. rubra*. It is a fact that both prokaryotic and eukaryotic organisms possess an inducible repair system, which confers cellular resistance to DNA damaging agents (Matsubara *et al*., 1987), and several genetically discrete DNA repair processes are known in yeast (Mitchel & Morrison, 1987). In addition, radio-resistance in yeast due to re-combinational repair capacity can be induced by variety of stresses including gamma radiation (Boreham & Mitchel, 1991). A strain of *Saccharomyces cerevisiae* when exposed to 54 KGy gamma rays, showed about 10% cell killing (Kaberi & Naresh, 1998), and exposure of this yeast to, 2.5 KGy of gamma rays did not affect cell survival (Khare *et al*., 1982). Also, many bacterial species other than yeasts were documented to be resistant to gamma-radiation from 2.0 - 4.4 KGy (Romanovskaya *et al*., 2002). The post-treatment

Table I. Survival percentages of two fungal yeasts irradiated with gamma rays

Dose RAD	*Hansenula anomala* CFU/mL 10^9	Survival %	Killing %	*Rhodotorula rubra* CFU/mL 10^9	Survival %	Killing %
0.0	2.1±0.1	100	-	2.2±0.3	100	-
10^5	1.9±0.2	90.5	9.5	2.1±0.09	95.5	4.5
$2x10^5$	1.6±0.07	76.2	23.8	1.9±0.07	86.4	13.6
$3x10^5$	1.3±0.06	62.0	38.0	1.7±0.06	77.3	22.7

Each value is the mean of 3 replica ± S.D.

Table II. Survival percentages of two fungal yeasts irradiated with fast neutrons

Dose RAD	*Hansenula anomala* CFU/mL 10^{10}	Survival %	Killing %	*Rhodotorula rubra* CFU/mL 10^{10}	Survival %	Killing %
0.0	3.5±0.3	100	-	24.7±0.5	100	-
0.03	2.9±0.1	82.9	17.1	18.1±0.7	73.3	26.7
0.3	2.2±0.07	62.9	37.1	16.9±0.8	68.4	31.6
3	1.9±0.06	54.3	45.7	12.4±0.7	50.2	49.8

Each value is the mean of 3 replica ± S.D.

Table III. The nucleotide sequences of the ten primers used for RAPD-PCR analysis

Primer	5` - sequence – 3`			
A6	GGT	CCC	TGA	C
A8	GTG	ACG	TAG	G
C20	ACT	TCG	CCA	C
D3	GTC	GCC	GTC	A
D5	TGA	GCG	GAC	A
D7	TTG	GCA	CGG	G
A19	CAA	ACG	TCG	G
B5	TGC	GCC	CTT	C
B10	CTG	CTG	GGA	C
C17	TTC	CCC	CCA	G

Table IV. DNA polymorphism induced by 3.0 KGy Gamma rays in *R. rubra* and *H. anomala*

Primer	*Hansenula anomala* Total Bands	No. % Polymorphism	*Rhodotorula rubra* Total Bands	No. % Polymorphism
A19	4	75.0	4	75.0
B5	9	33.3	10	30.0
B10	8	37.5	6	66.7
C17	11	45.5	11	63.6
A6	8	37.5	4	25.0
A8	10	40.0	10	40.0
C20	7	57.1	13	31.1
D3	8	50.0	9	55.6
D5	9	66.7	12	50.0
D7	4	75.0	2	50.0
X (MEAN)		51.8		47.9

repair process following gamma treatment was reported to be modified via different pathways (Kshiti & Rao, 2001; Mercier *et al*., 2001; Craig *et al*., 2001).

Irradiation of DNA in aqueous solutions or in cells results in different mutational spectra, indicating that in both situations, different patterns of DNA lesions are induced. One of the causes for these different types of lesions might be the formation of secondary organic radicals. These secondary radicals react with oxygen, thus forming peroxyl radicals, which can be very harmful to DNA and which may therefore induce DNA damage or DNA polymorphism leading finally to mutations (Wijker *et al*., 2000). It appears that ionizing radiation induced mutation spectrum after irradiation of DNA in aqueous solution in more comparable manner to the intracellular radiation induced mutation spectrum in *E. coli* cells (Wijker *et al*., 2000). The effects of ionizing radiation on yeast including induction of conversion resulting from re-combinational DNA repair, mutation resulting from error prone DNA repair, lesions induced in DNA, cell killing and other genetic events was reported by several a (Mitchel & Morrison, 1984; Unrau, 1986; Kiefer *et al*., 1988; Deorukhakar & Rao, 1999). A genomic instability in *Saccharomyces cerevisiae* upon exposure to gamma radiation was detected by (Zyuzikov & Petin, 1996). Moreover, increased forward mutation frequencies and low colony forming efficiencies were observed form gamma radiation exposure. However, an increase in intrachromosomal recombination was detected in a culture derived from cell surviving gamma irradiation for as many as 50 generations after the exposure (Richard & robert, 2001).

The content of carotenoid in the cell may also influence the radio-resistance mechanism. Hyper-producing carotenoid yeast survived gamma irradiation by scavenging oxygen radicals (NamKyu *et al*., 2002).

This study clearly showed great difference in the efficiency of both sources of radiation in cell killing. The low LET rays (gamma irradiation) are more efficient in inducing radio resistance to killing than high LET (fast neutrons). This may be due to DNA-SSBS that produced by

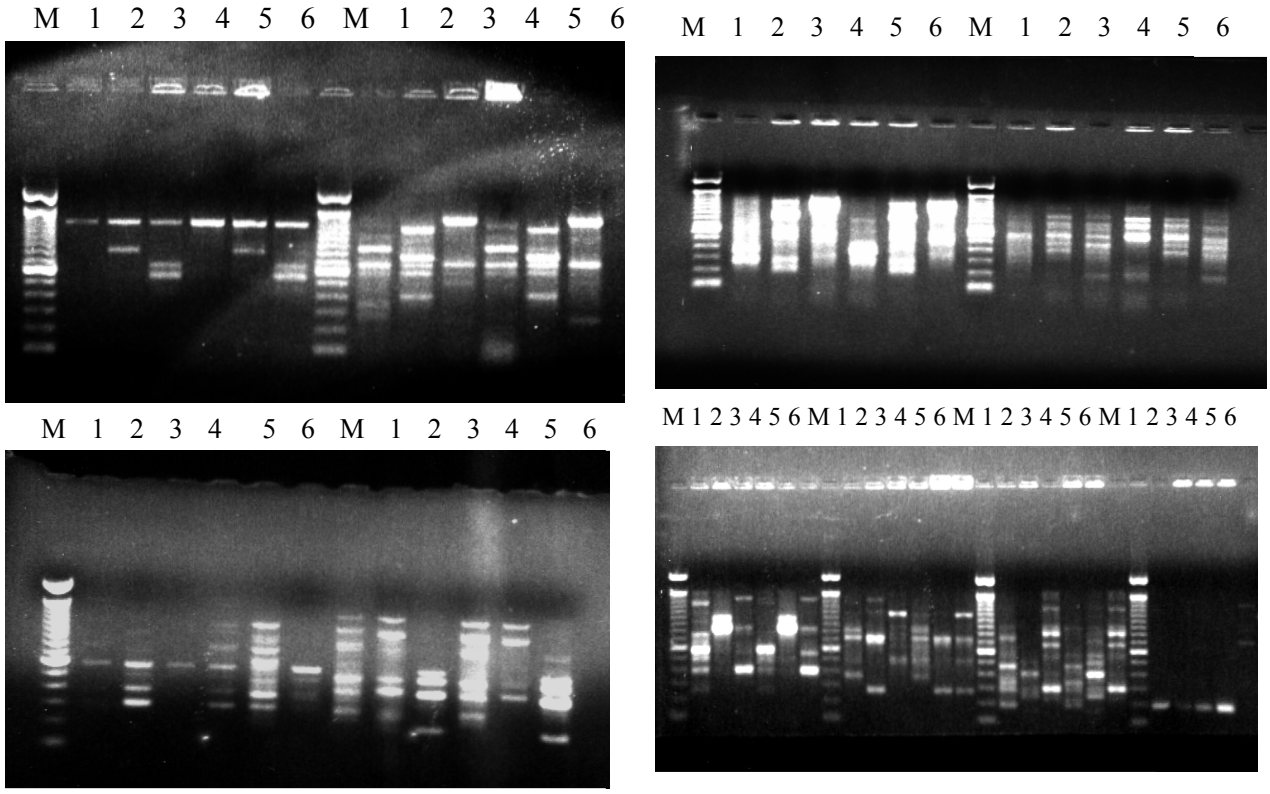
Table V. DNA polymorphism induced by 3 RAD fast neutrons in *R. rubra* and *H. anomala*

Primer	*Hansenula anomala* Total Bands	No. % Polymorphism	*Rhodotorula rubra* Total Bands	No. % Polymorphism
A19	3	66.6	3	66.6
B5	12	50.0	14	50.0
B10	10	50.0	7	71.4
C17	11	45.5	10	60.0
A6	12	58.3	9	66.7
A8	10	40.0	11	45.5
C20	7	57.0	12	16.7
D3	6	33.3	8	50.0
D5	10	70.0	9	33.3
D7	3	66.6	2	50.0
X (MEAN)		53.7		51.02

Table VI. Number of Amplified DNA fragments produced by ionizing radiation

Gamma rays ($3 x 10^5$ RAD)		Fast Neutrons 3 RAD
H. anomala	78	84
R. rubra	81	85

Fig. 1. RAPD Profiles of *Rhodotorula rubra* and *Hansenula anomala* irradiated with 3 MRAD* of gamma rays and 3 RAD of fast neutrons using different primers* MRAD = 10^6 RAD



gamma rays are more important in the induction of radio-resistance than DNA-DSBS that are generated by fast neutrons (Boreham & Mitchel, 1991).

DNA-polymorphism revealed by RAPD. One dose which gave a higher *killing percentage* in case of the two fungal yeasts using both types of ionizing radiation was chosen to study its effect on DNA polymorphism, using RAPD analysis. This dose was 3 M RAD of gamma rays and 3 RAD of fast neutrons. Analysis of 10 RAPD primers [nucleotide sequences for the 10 primers are shown in (Table III) leading to a band profiles with a number of amplified DNA fragments shown in (Table IV) for gamma rays and in (Table V) for fast neutrons.

DNA Polymorphism may appear as shifts in band migration or missing bands, or differences in band intensities. The polymorphism percentages ranged from 23.1 up to 75.0 in *R. rubra* and from 33.3 up to 75.0 in *H. anomala* when using 3 M RAD of gamma rays. This means that the means of polymorphism percentages reached 47.9 and 51.8 in *R. rubra* and *H. anomala,* respectively. However, the polymorphism percentages ranged from 16.7 up to 66.7 in *R. rubra* and from 33.3 up to 70.0 in *H. anomala* when 3 RAD of fast neutrons was used (The means of polymorphism percentages for the 10-primers reached 51.02 and 53.7 in *R. rubra* and *H. anomala,* respectively). Again, fast neutrons were more efficient than gamma rays in inducing DNA-polymorphism.

RAPD profiles for the two fungal yeasts when exposed to the ionizing radiation are shown in (Fig. 1). The number of amplified DNA fragments that are generated by ionizing radiation are also varied depending on type of ionizing radiation used (Table VI). 3 M RAD of gamma radiation induced 81 and 78 amplified fragments in *R. rubra* and *H. anomala*. Whereas 3 RAD of fast neutrons resulted in 85 and 84 amplified fragments in the two fungal yeasts. Finally, these results revealed that RAPD technique can be easily used to demonstrate DNA polymorphism.

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