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Genetic improvement of lignin peroxidase enzyme production from *Phanerochaete chrysosporium*

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Abstract : Four different *Phanerochaete chrysosporium* strains collected and evaluated using two fermentation media and three incubation periods (5, 7 and 9 days) proved that the strain *P. chrysosporium* (PhC3) was the highest lignin peroxidase (LiP)producer and the medium 1(FM_1) combined with the incubation period 7 days were the best condition for LiP production. The highest LiP producer mutant, obtaining after UV-mutagenesis, was Kh-UV-4 since it showed 115 percent production higher than the original strain.Furthermore,the superior mutant Kh-EMS-9**,** obtaining after EMS-mutagenesis, was produced 171.25 percent production higher than the original strain. Moreover, the first and second crosses were carried out,on the intraspecific protoplast fusion level, between high LiP producer mutants. The enhancement of LiP productivity by these crosses reached up to 202.50 and 251.25 percent higher than the original strain (PhC3) for the fusants Kh-C1-4and Kh-C2-1, respectively.Three15-mer random primers were applied using RAPD technique to detect the molecular variation between three mutants and threefusants compared to their original strain. The results showed that many differences in RAPD banding patterns profile were detected as a result of mutagenic treatments and protoplast fusion. These differences confirmed the evidence of genetic variation in genomes after the mutagenic treatments and protoplast fusion crosses.
Key words : Improvement, lignin peroxidase, *P.chrysosporium*, Mutants, Fusants, RAPD-PCR.

Introduction

Next to cellulose, lignin is the most abundant natural polymeric carbon source in the world. Development of lignin degradation process has considerable potential for the production of a variety of chemicals, which could serve as replacement for various petroleum-derived chemicals[1]. White-rot fungi, the only microorganisms able to efficiently degrade lignin, play a very important role in the global carbon cycle. The extracellular lignin peroxidase (LiP) secreted by White-rot fungus of *P.chrysosporium* is the key enzyme in lignin degradation[2]. Random mutation was introduced for white rot fungi using X rays, UV-irradiation and Gamma irradiation[3,4,5,6].In some cases, such as cellulase-deficient mutants of *P.chrysosporium*, an increase in ligninolytic activity was reported[2]. On the other hand, Protoplast fusion an important tools in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi[7]. As conventional hybridization, protoplast fusion can be performed intraspecifically[8], interspecifically[9], intergenerically[10].

In the present study, the ability of different strains of *P. chrysosporium* in the production of extracellular lignin peroxidase enzyme presence were monitored in production media. Potential for genetic manipulations, including sensitivity to antibiotics, Physical and chemical mutagens, intra-protoplast fusion, genetic analysis of

products randomly amplified polymorphic DNA (RAPD) technique to detect the molecular variations and their effect on productivity were evaluated.

Materials and Methods:

Microorganisms:

The four different strains of *Phanerochaete chrysosporium* were used. The cultures used were: white-rot fungi, *P.chrysosporium* NRRL 6361(PhC1) and *P.chrysosporium* NRRL 6359(PhC2)in addition of two local isolates(PhC3 and PhC4) of *P.chrysosporium* obtained from Genetics and Cytology Department, National Research Centre (NRC), Egypt, were used.

Culture conditions:

All incubations were carried out at 28^{o}C stationary in the dark. The *P. chrysosporium* strain was inoculated onto 10 ml growth medium(glucose, 10; yeast extract,2.5; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.02;K_2HPO_4, 0.02; $MgSO_4$, 0.01 g/L)in a 100 ml Erlenmeyer flask, and then cultivated for 5 days for pre-cultivation. 50 ml of the two Fermentation culture media (FM1 and FM2) according to **Asther et al.,**[11]and **Aloui et al.,**[12], respectively, with some modifications. (**FM1:**glycerol, 10; asparagine, 1; ammonium nitrate, 0.5; yeast extract, 0.1; Lignin, 0.1 g/L; oleic acid, 0.4 g/Lveratryl alcohol, 0.4 mM; pH was adjusted to 5.5and **FM2:**KH_2PO_4, 1.0; $CaCl_2$, 0.07;$MgSO_4.7H_2O$, 0.35; $FeSO_4.7H_2O$, 0.035; $ZnSO_4.7H_2O$, 0.023; $CuSO_4.5H_2O$, 0.0035; Lignin, 0.1 g/L; veratryl alcohol, 0.4 mM and the pH was adjusted by 20 mM disodium tartrate to 6.5) was added to a 250 ml Erlenmeyer flask, followed by inoculation of 10ml of the above inoculum and cultivation for 5-9 days at 37^{o}C.

Assay of lignin peroxidase enzyme:

The enzyme productivity was determined in the culture supernatant obtained by centrifugation for 5 min. at 5000 rpm under cooling in an Eppendorf tube. A half ml of the clear supernatant was used to assay the enzyme activity. LIP activity was measured according to **Tien and Kirk** [13], with one unit (U) representing 1 µmol veratryl alcohol oxidized to veratryl aldehyde per minute at pH 3.0 and 30 ^{o}C.

UV-Mutagenesis:

Spore suspension of the selected strain was prepared in saline solution (0.85%, w/v, NaCl containing a drop of Tween 80) from 7 days old slant and irradiated with Philips TUV-30-W-245 nm Lamp, type No. 57413-P/40 for 4; 8; 12 and 16 min at a distance of 20 cm. The treated spores were kept in a dark for 2 hours to avoid photoreactivation repair. Then, the treated spores were diluted and spread onto culture medium containing 0.1% (v/v) Triton X-100 as a restriction factor for radial colonies grow that 28°C for five days. The growing colonies were transferred onto slants for further studies.

EMS-Mutagenesis:

Spore suspension of the used microorganism was prepared in phosphate buffer (0.1M, pH 7) containing a drop of Tween 80 and treated with 50 mM of EMS for 60 min. Then the treated spores were diluted and spread onto culture medium containing 0.1% (v/v) Triton X-100 as a restriction factor for radial colonies growthat 28°C for five days. The growing colonies were transferred onto slants for further studies.

Protoplast formation:

Five day-old mycelia of parental strains were transferred onto 50 ml of malt extract broth (MEB medium) in a 250 ml Erlenmeyer flask placed on a shaker with the agitation speed of 200 rpm, at 28ºC for 1-2 days. Protoplasts were separated from the mycelia using the method from **Larraya et al.,**[14] by transferring of mycelia onto 3 ml of the sterilized protoplast isolation buffer in a test tube and shaking it at 100 rpm at room temperature for 4 h. The mycelial remnants were removed by filtration through a 0.45 mm pore-size membrane and the suspended protoplasts were collected by centrifugation at 500 xg for 15 min. The protoplasts were then washed twice with the osmotic stabilizer (0.7 M manitol, pH6) and were finally suspended in 5 ml of the osmotic stabilizer. Protoplasts obtained were counted using a hemocytometer.

Protoplast fusion:

One milliliter of each of the freshly prepared protoplasts of parental strains was mixed in a test tube, and centrifuged at 1000 xg for 10 min. The supernatant was rinsed off and 1 ml of sterilized protoplasting buffer containing 30% PEG was added to the protoplasts in the test tube and incubated for 20 min by shaking the tube every 5 min. The mixed protoplasts were collected by centrifugation at 1000 xg for 10 min. and then washed twice with 0.7M manitol using the method from **Dhitaphichit and Pornsuriya**[15]. Treated protoplast pellets were resuspended in 1 ml of osmatically balanced phosphate buffer and diluted appropriately.

Protoplast regeneration:

Protoplast solution was diluted to $1x10^4$ protoplasts/ml and 0.1 ml of suspension was used for protoplast regeneration by culturing it on MEB agar plate osmotically supported by 0.7 M manitol. Plates were incubated at 28 ˚C until colonies were grown on the surface of plates. The grown colonies were considered as complementary fusants. They were transplanted and sub-cultured several times onto selective and nonselective media before further studies. Fusion frequency was expressed as the ratio of the number of colonies formed on selective and nonselective media. **Molecular analysis of new superior mutants by PCR:**

Ready-To-Go PCR Beads (Amersham, Pharmacia Biotech. No. 27-9555-01) were used for PCR experiments. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Three different primers were used in the present study. The first primer (P1) sequence was 5'-CAT ACC CCC GCC GTT-3'. The second primer (P2) sequence was 5'-GTG TTG TGG TCC ACT-3'. The third primer (P3) sequence was 5'-TGA GTG GTC TAC GTG-3'. All primers were supplied by Operon Technologies Company, Netherlands. To each 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. The amplification protocol was carried out as follows: Denaturation at 95^{o}C for five min. Thirty-five cycles each consists of the following segments: Denaturation at 95^{o}C for one min; primer annealing for two min. according to GC ratio of each primer and incubation at 72^{o}C for two min. for DNA polymerization. At the end, hold the PCR at 4^{o}C till analysis. The amplified DNA products from RAPD analysis were electrophorated on 1.0% 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 (Vivantis # NL 1407- Malaysia) and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using Gel Documentation System with UV Transeliminator.

Results and discussions:

The present study was designed to construct new higher LiP producers of *P. chrysosporium*. To achieve this goal, four different strains of *P. chrysosporium* were firstly collected as mentioned in Materials and Methods. All of the collected strains (Table 1) as well as two different fermentation media (FM) combined with three incubation periods were evaluated to determine the best strain and conditions for LiP production as the following:

A. Evaluation of the collected strains and fermentation media:

Table (1) presented the effect of two different fermentation media (FM_1 and FM_2) along with the incubation periods (5, 7 and 9 days) on LiP productivity of the collected *P. chrysosporium* strains.

Data in Table (1) clearly showed that the fermentation medium No. 1 (FM_1) proved to be the best one since the alltested strains produced higher amounts of LiP production on this medium following the different incubation periods. The highest records of LiP productivity was 80 Units.L-^{1}which were obtained from the original strain *P. chrysosporium* (PhC3) for 7 days. The following strain for LiP productivity was strain PhC4 on the FM_1. On the other hand, the lowest record of LiP production following 5 days of incubation was 62 Units.L-^{1} which was obtained from the strain PhC2 (*P.chrysosporium*NRRL6359) on FM_2.

From the foregoing results out of the four tested strains, *P.chrysosporium*(PhC3)proved to be the highest LiP producer on FM_1 in comparison with the other tested strains. So, it was selected to be the original strain for all of the following genetic techniques to improve LiP production on FM_1.

Table (1): Effect of different fermentation media and incubation times on LiP production of the original strains cultured in shake flasks as a batch fermentation.

Strain code No.	FM1			FM2		
	5d*	7d	9d	5d	7d	9d
PhC1	71**	75	74	65	65	64
PhC2	70	73	73	**62**	63	65
PhC3	72	**80**	78	68	70	71
PhC4	71	77	77	69	70	70

***d=days, ** Units.L-^{1}**

B. Mutation induction and LiP productivity:

Table (3) presents the LiP production of 33 randomly selected isolates out of the survived mutants following the exposure of *P.chrysosporium*(PhC3)to UV-light for 15 min compared to the untreated original strain. These results indicated that the majority of the tested mutants produced LiP lower than their original parental strain. Meanwhile, four mutants, i.e., Kh-UV-3, Kh-UV-18 and Kh-UV-32 proved to have a little bit more efficiency LiP productivity than the original strain. The highest LiP producer mutant was Kh-UV-4 since it showed 115percent production higher than the original strain. The following mutant for LiP productivity was Kh-UV-25 which produced 103.75 percent production higher than the original strain.

Table (4) presents the LiP productivity of randomly selected 33 mutants following exposure of the parental strain to EMS-mutagen for 60 min. compared to its parental strain productivity. The obtained results showed an opposite trend to those obtained following UV-light since the majority (18 out of 33) of the tested mutants produced LiP higher than their original parental strain. The highest record of the LiP production was 217 Units.L-^{1} (with 171.25% more than the original untreated strain) which was obtained from the mutant Kh-EMS-9. Meanwhile, 17 mutants produced LiP higher than the original strain but lower than the superior mutant Kh-EMS--9 following the same treatment. On the other hand, 14 mutants of the tested 33 ones lost some of their productivity in comparison to their parental strain. Onemutant Kh-EMS-3 produced LiP at the same of the original strain.

Using UV-light as a mutagenic agent has been applied as the method of choice for increasing the LiP productivity[16, 17, 18]. Since, it UV-induced mutants were more stable through long term of generation and subculturing[19]. In addition, UV-light also induced tolerance to different environmental stresses and changes in protein synthesis [20]. Furthermore, UV-light is safety usage and causing no pollution.

The obtained results were in agreement with those obtained by[17]**.** They improved LiP production by *P. chrysosporium* using UV-mutagenesis and used a positive selection procedure, based on lignin peroxidase activity, for obtaining overproducing mutants after UV-mutagenesis. The mutants described here produce much higher ligninase activity than wild-type BKM-F-1767. Moreover, different variabilities of enzymatic activities in ligninolytic fungi *Pleurotus ostreatus* and *Lentinus tigrinus* after protoplasting and UV-mutagenization were obtained by **Homolka et al.,**[21].Also, mutant strains of *Pleurotus ostreatus* with the objective of enhancing their ligninolytic activities in white rot fungi were obtained by **Vijayaet al.,**[22] and **Ibrahimet al.,**[23].

On the other hand, the strain modification for hyper-production of extracelluar LiP enzyme using *P. chrysosprium* is achieved also by EMS-mutagenesis. Through EMS-mutagenesis, introduction of point mutations into different genes encoding lignocellulose degrading enzymes can occur but mutations may be silent, nonsense or point mutations where no improvement occurs. Also, chemical mutagenesis results in deleterious mutations and many mutants need to be screened before a mutant with improvement is found[24]**.** Furthermore, Muhammad et al, [25]increased the yield of MnPs from *T.veriscolor* IBL-04 by chemical mutagenesis. Compared with the native strain, the activity of MnPs from two genetically modified mutants, EB-60 and EMS-90, was increased by 29.6% and 32.5%, respectively. The mutant MnPs were tolerant to a wider Ph and temperature range than the native MnP. The high MnP production in culture filtrates of mutated strains may be attributed to over expression of the mnp-1 gene caused by the chemical mutagens.

Table (3): LiP productivity of different mutants obtained following exposure of *P.chrysosporium*(PhC3)to UV-light for 15min.

Strain No.	LiP ($U.L^{-1}$)	% to W.T	Strain No.	LiP ($U.L^{-1}$)	% to W.T
W.T(PhC3)	80	100.00	Kh-UV-17	55	68.75
Kh-UV-1	95	118.75	Kh-UV-18	82	102.50
Kh-UV-2	70	87.50	Kh-UV-19	71	88.75
Kh-UV-3	85	106.25	Kh-UV-20	55	68.75
Kh-UV-4	**172**	**215.00**	Kh-UV-21	52	65.00
Kh-UV-5	62	77.50	Kh-UV-22	92	115.00
Kh-UV-6	86	107.50	Kh-UV-23	65	81.25
Kh-UV-7	73	91.25	Kh-UV-24	78	97.50
Kh-UV-8	44	55.00	Kh-UV-25	**163**	**203.75**
Kh-UV-9	79	98.75	Kh-UV-26	67	83.75
Kh-UV-10	92	115.00	Kh-UV-27	75	83.75
Kh-UV-11	65	81.25	Kh-UV-28	65	81.25
Kh-UV-12	65	81.25	Kh-UV-29	62	77.50
Kh-UV-13	73	91.25	Kh-UV-30	140	175.00
Kh-UV-14	78	97.50	Kh-UV-31	63	78.75
Kh-UV-15	125	156.25	Kh-UV-32	82	102.50
Kh-UV-16	63	78.75	Kh-UV-33	75	83.75

C. Protoplast fusion and lactic acid productivity:

As mentioned earlier, protoplast fusion was the main protocol for exchanging genetic material between two cell types and subsequently obtaining the new gene recombinants towards the isolation of higher LiP producing fusant(s).

Table (4):LiP productivity of different mutants obtained following exposure of *P. chrysosporium* (PhC3) to EMS for 60 min.

Strain No.	LiP ($U.L^{-1}$)	% to W.T	Strain No.	LiP ($U.L^{-1}$)	% to W.T
W.T(PhC3)	80	100.00	Kh-EMS-17	85	106.25
Kh-EMS-1	65	81.25	Kh-EMS-18	62	77.50
Kh-EMS-2	76	95.00	Kh-EMS-19	141	176.25
Kh-EMS-3	80	100.00	Kh-EMS-20	85	106.25
Kh-EMS-4	126	157.50	Kh-EMS-21	85	106.25
Kh-EMS-5	72	90.00	Kh-EMS-22	74	92.50
Kh-EMS-6	87	108.75	Kh-EMS-23	115	143.75
Kh-EMS-7	97	121.25	Kh-EMS-24	165	206.25
Kh-EMS-8	76	95.00	Kh-EMS-25	118	147.50
Kh-EMS-9	**217**	**271.25**	Kh-EMS-26	77	96.25
Kh-EMS-10	92	115.00	Kh-EMS-27	73	91.25
Kh-EMS-11	69	86.25	Kh-EMS-28	85	106.25
Kh-EMS-12	93	116.25	Kh-EMS-29	72	90.00
Kh-EMS-13	73	91.25	Kh-EMS-30	56	70.00
Kh-EMS-14	69	86.25	Kh-EMS-31	175	218.75
Kh-EMS-15	126	157.50	Kh-EMS-32	82	102.50
Kh-EMS-16	**193**	**241.25**	Kh-EMS-33	65	81.25

C.1. Response of the original strain (PhC3) and selected mutants to different heavy metals:

In order to investigate the effect of intraspecific protoplast fusion on LiP production, six original strains (Materials and Methods), three UV treated mutants and two EMS treated mutants were selected to determine their heavy metals resistance or sensitivity as an additional selective marker.

Table (5) presents the selected strains and mutants response to 5 different types of heavy metals. Results showed that, the parental strain (PhC3) was resistant to Cd, As, and Mc. Meanwhile, they were sensitive to the other heavy metals. In addition, the mutant Kh-EMS-31 showed the highest level of heavy metals resistance since it was resistant to four heavy metals as follows: Cd, As, Se and Mc. The response of the rest of mutants to heavy metals as follows: The mutants Kh-UV-4 and Kh-EMS-16 were resistant to As, Co, and Mc and sensitive to the others. The mutants Kh-UV-30andKh-EMS-24 were resistant only to Mc and sensitive to the others. Finally, mutant Kh-EMS-9 was resistant only to Co and sensitive to the others. The above results showed clearly that the effects of UV and EMS were not only mutagenic but also have regulatory effects on LiP productivity and heavy metals response.

C.2. Preparation of *P. chrysosporium* protoplasts:

According to the conditions described under Materials and Methods, enzymatic treatments and subsequently examination of the treated fungal mycelium with phase-contrast microscope showed that the gradual degradation of fungal cell wall started after the addition of Lysing enzyme as shown in Figure (1) which described the protoplasting steps.

C.3. LiP productivity of fusants obtained after protoplast fusion:

Aliquots (500 µl) of both parental protoplast suspensions were used for protoplast fusion and regeneration experiments as mentioned under Materials and Methods. Table (6) presents the LiP productivity of the parents and the protoplast fusants which were obtained following the different crosses at the level of intraspecific protoplast fusions.

Table (5): Heavy metals response of different superior mutants obtained after mutagenesis of *P.chrysosporium* (PhC3).

Original st. and mutant No.	Heavy metals (mg/L)				
	Cd (400)	As (500)	Co (300)	Se (150)	Mc (75)
W.T (PhC3)	+	+	-	-	+
Kh-UV-4	-	+	+	-	+
Kh-UV-25	+	-	-	+	+
Kh-UV-30	-	-	-	-	+
Kh-EMS-9	+	-	-	-	-
Kh-EMS-16	-	+	+	-	+
Kh-EMS-24	-	-	-	-	+
Kh-EMS-31	+	+	-	+	+

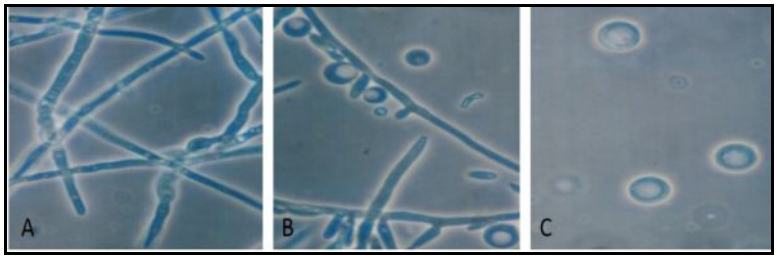

Figure (1):Photomicrographs of *P. chrysosporium* protoplasts (B) and (C) in comparison with normal mycelia (A).

Results in this table showed that, cross 1 was carried out between two high LiP producing isolates, i.e., Kh-UV-4 and Kh-EMS-9. The obtained results showed that, five out of the six fusants were higher LiP producers than both parents. For instance, the highest fusantKh-C1-4produced 202.50percent more LiP than the original wild type strain (PhC3) and at the same time represents 11.52percent more LiP than its higher producer parent. Meanwhile, only one fusant (Kh-C1-3) showed less LiP production than both two parents although they still higher than the original parental strain(PhC3).

The second cross was conducted also between protoplasts of two high producer mutants, i.e., Kh-EMS-9and Kh-EMS-16. Six fusants were obtained and all of them proved to be higher LiP producer than the original strain. Four out of the six ones exhibited higher LiP productivity than their higher producer parents. The excellent fusant wasKh-C2-1which produced 251.25percent LiP more than the original strain (PhC3)and at the same time represents 29.49percent more than the higher parent (Kh-EMS-9).

Protoplast fusion technique or genome shuffling is an alternative strain improvement. In this approach, protoplasts are obtained by the removal of the cell wall then fused together for random recombination of the genome to occur[26, 27].Protoplast fusion has been successfully used to enhance degradation of lignocellulose. For example, cellulase and xylanase activity was improved compared to parental strains in studies where inter-strain genome shuffling between *Penicillium echinulatum* and *Trichodermaharzianum*[28], and intra-strain improvement as in *Trichoderma reesei*[29] and *Trichoderma reesei* QM 9414 fusions[30] and intra-strain improvement as in *Pleurotus ostreatus* strains by protoplast fusion[31].

Table (6):LiP productivity of different fusants obtained after two different protoplast fusion crosses.

Crosses	Parents and fusants No.	LiP production ($U.L^{-1}$)	LiP % to higher parent	LiP % to W.T
	W.T(PhC3)	80	36.87	100.00
Cross 1	Kh-UV-4	172	79.26	215.00
	Kh-EMS-9	217	100.00	271.25
	Kh-C1-1	196	90.32	245.00
	Kh-C1-2	238	109.68	297.50
	Kh-C1-3	167	76.96	208.75
	Kh-C1-4	242	**111.52**	**302.50**
	Kh-C1-5	189	87.10	236.25
	Kh-C1-6	227	104.61	283.75
Cross 2	Kh-EMS-9	217	100.00	271.25
	Kh-EMS-16	193	88.94	241.25
	Kh-C2-1	281	**129.49**	**351.25**
	Kh-C2-2	214	98.62	267.50
	Kh-C2-3	235	108.29	293.75
	Kh-C2-4	278	128.11	347.50
	Kh-C2-5	225	103.69	281.25
	Kh-C2-6	205	94.47	256.25

D. Molecular analysis of mutants and fusants:

An attempt was conducted to evaluate the genetic effects of mutageneic treatments and protoplast fusion on the DNA nucleotide sequence of the obtained mutants and fusants compared to the original strain was also considered. Three 15-mer random primers were applied using randomly amplified polymorphic DNA (RAPD) technique to detect the molecular variations between three and three fusants compared to their original strain (PhC3).

Results in Figure (2) showed clear differences at bands number and size between the original strain and its derivatives using primer No. 1. Using primer (P1), it was clearly noticed that two amplified bands were occurred when the original strain DNA was used as a template. The bands sizes were 600 and 850 bp (very faint). Three amplified bands 600, 850 bp (very faint) and 1200 bp (very faint) were occurred when the Kh-UV-

4 DNA was used as a template (Lane 2). On the other hand, two mutants (Kh-EMS-9 and Kh-EMS-16) exhibited the same banding patterns of the amplified regions (Lanes 3, 4). Five faint bands were detected with base pair sizes of 300, 400, 550, 650 and 900. On the other hand, the fusant (Kh-C1-4) did not contain the complementary sequences of the primer (P1),since none any amplified bands were detected (Lane 5). The fusant (Kh-C2-1) exhibited the highest number of amplified bands, since ten amplified bands were detected (lane 6). Finally, the fusant (Kh-C2-4) exhibited four of amplified bands, with base pair sizes of 300, 400, 600 and 1300 (Lane 7).

The results presented in Figure 3 presented the random amplified banding patterns of the original strain and the tested mutants and fusants when primer (P2) was used. Two bands were detected for the original strain with sizes of 1600and 2000 bp (Lane 1). Moreover, an additional band with size of 1400 bp was occurred (Lanes 2, 3, 6) for the three strains (Kh-EMS-9, Kh-EMS-16 and Kh-C2-1). Also, an additional band with size of 1300 bp was occurred (Lanes 3, 6) for the three strains (Kh-EMS-16 and Kh-C2-1). On the other hand, the amplified band with size of 2000 bp was detected in all tested mutants and fusants.

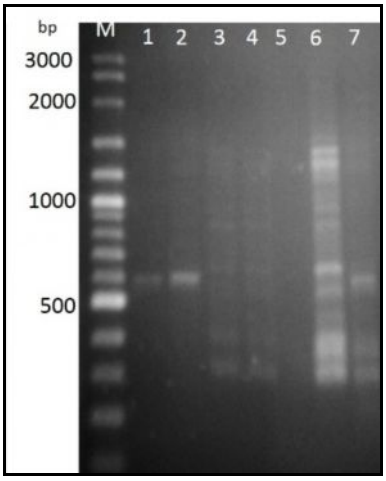

Fig. 2: Photograph of DNA amplified banding profiles based an RAPD for three superior mutants and fusants compare with original strain, PhC3 (Lane 1) using primer (P1) against VC100 bp plus DNA ladder Vivantis # NL 1407- Malaysia (lane M).Mutants sequence as follows: (Lanes 2 to 4), Kh-UV-4, Kh-EMS-9, Kh-EMS-16 and fusants sequence as follows: (Lanes 5 to 7), Kh-C1-4, Kh-C2-1, Kh-C2-4.

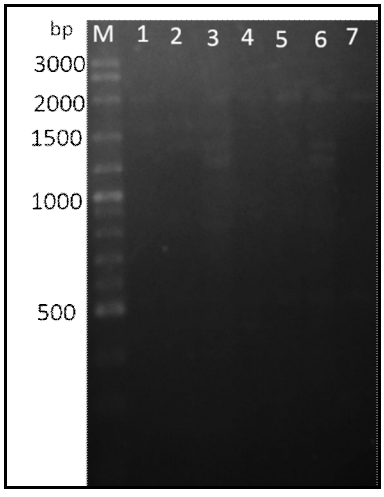

Fig. 3: Photograph of DNA amplified banding profiles based an RAPD for three superior mutants and fusants compare with original strain, PhC3 (Lane 1) using primer (P2) against VC100 bp plus DNA ladder Vivantis # NL 1407- Malaysia (lane M). Mutants sequence as follows: (Lanes 2 to 4), Kh-UV-4, Kh-EMS-9, Kh-EMS-16 and fusants sequence as follows: (Lanes 5 to 7), Kh-C1-4, Kh-C2-1, Kh-C2-4.

Using primer(P3) against the selected mutants and fusants (Figure 4) exhibited extreme different random amplified banding patterns between all of the tested strains from one side and their original parental strain from the other side. Two bands were detected for the original strain with sizes of 500 and 1750 bp (Lane 1). Three very faint amplified bands 400, 1400 bp and 1750 bp were detected when the DNA of mutant Kh-UV-4 was used as a template (Lane 2). On the other hand, the mutant Kh-EMS-9 exhibited two amplified bands with sizes of 900 and 1200bp (Lane 3). The mutant Kh-EMS-16 exhibited three amplified bands with sizes of 1000, 1200 and 1750 bp (Lane 4).The fusant (Kh-C1-4) exhibited the same amplified bands of mutant Kh-EMS-16, butan additional band with size of 500 bp was occurred (Lane5).The fusant (Kh-C2-1) exhibited the highest number of amplified bands, since five amplified bands were detected (lane 6). Finally, the fusant (Kh-C2-4) exhibited only one amplified band, with base pair size of 1500(Lane 7).

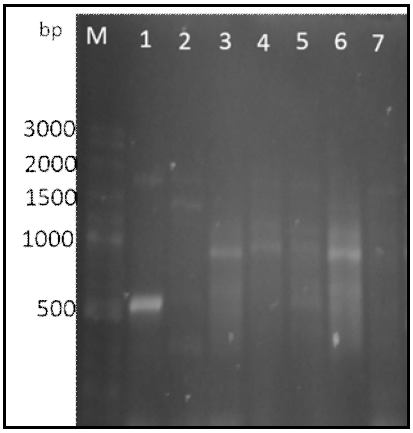

Fig. 4: Photograph of DNA amplified banding profiles based an RAPD for three superior mutants and fusants compare with original strain, PhC3 (Lane 1) using primer (P3) against VC100 bp plus DNA ladder Vivantis # NL 1407- Malaysia (lane M). Mutants sequence as follows: (Lanes 2 to 4), Kh-UV-4, Kh-EMS-9, Kh-EMS-16 and fusants sequence as follows: (Lanes 5 to 7), Kh-C1-4, Kh-C2-1, Kh-C2-4.

The above differences in RAPD profile confirmed the evidence of genetic variation in genomes after the mutagenic treatments and protoplast fusion crosses.

The obtained results showed excellent harmony with those reported by **Schlick et al., [32]**and **Lee et al., [33]**. They demonstrated that trend was found in the mutants of *T. harizianum* and *Pleurotus ostreatus* induced by gamma-ray radiation and analyzed by RAPD.

Moreover, **Khattab and Abd-El Salam [34]**studied the molecular analysis of mutation by RAPD-PCR analysis in *Mucor racemosus* and they generated a total of 27 distinct fragments, 8 (29.63%) of them being polymorphic after the amplification reactions with two primers. These differences in RAPD profiles confirmed the evidence of genetic variations of mutants and *M. racemosus* genome after UV-mutagenesis. On the other hand, **Capelari and Fungaro[35]** and **Shoukry, et al., [36]**suggested that the genetic variation among closely related strains could be detected by RAPD-PCR technique.

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