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Genetic Improvement of the β-oxidation Pathway in the Yeast *Yarrowia lipolytica* to Increase the Production of γ-decalactone

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Abstract: The γ –decalactone - a peach aroma compound - production by *Yarrowia lipolytica* is of great interest due to its increasing acceptability, especially in the food industry, in comparison with similar products produced by chemical synthesis. The purpose of this work is to enhance the biotransformation of castor oil into γ-decalactone using lipase produced by genetically improved strains of Y. *lipolytica*. Ultraviolet (UV) ray was used in the treatment of *Y. lipolytica* for increasing its lipase and γ-decalactone production. The results demonstrated that lipase enzyme was increased up to three times as the wild type strain after UV treatment of *Y. lipolytica.* Also, results indicated γ-decaactone production was apparently increased after application of UV mutagenesis. The γ-decalactone production of some UV mutants was as fivefold of wild type strain. In general, data indicated very highly positive correlation between the activity of lipase produced and the amount of γ –decalactone production by the superior UV-mutants. On the other hand, the application of the random amplified polymorphic DNA (RAPD) technique by polymerase chain reaction (PCR) on some selected excellent mutants lead to correlate the genetic characteristics of the excellent mutants with the results obtained from the biotransformation experiments in comparison with the wild type. Differences in RAPD patterns confirmed the evidence of genetic variability induced in *Y. lipolytica* genome after UV-treatments.

Key words: γ-decalactone, Lipase, *Yarrowia lipolytica*, UV-mutagenesis, GC, RAPD-PCR.

INTRODUCTION

Since the end of the last century people have become more concerned about their health. Natural foods are a very common requirement nowadays, including mainly natural ingredients. This means that natural flavour compounds could not be obtained only by extraction from plants anymore because their great demand. The main objective is not only the creation of natural flavours, but also the development of new technologies. Food legislation from many countries recognizes that natural flavours can be obtained by biotechnological methods. Three principal techniques of biotransformation can be distinguished in the followed way: (1) use of enzymes, (2) use of microorganisms, (3) plant cells and culture of tissues. Application of enzymes is the most used technique of biotransformation. The use of lipase for direct esterification reactions in free-solvent media is a good alternative to produce flavour compounds for the food industry. Several studies show high potential for the industrial use of terpene modifications (Melo *et al*., 2005).

An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases. The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Lesser amounts of lipases are used in oleochemical transformations (Bornscheuer, 2000). Lipases can play an important role in the processing of γ- linolenic acid, a polyunsaturated fatty acid (PUFA); astaxanthine, a food colorant; methyl ketones, flavor molecules characteristic of blue cheese; 4-hydroxydecanoic acid used as a precursor of γ -decalactone, a fruit flavour; dicarboxylic acids for use as prepolymers; interesterification of cheaper glycerides to more valuable forms (e.g., cocoa butter replacements for use in chocolate manufacture) (Undurraga *et al*., 2001); modification of vegetable oils at position 2 of the triglyceride, to obtain fats similar to human milk fat for use in baby feeds; lipid esters including isopropyl myristate, for use in cosmetics; and monoglycerides for use as emulsifiers in food and pharmaceutical applications.

Lactones are widely distributed in foods and beverages as aroma compounds. Among these compounds, γ -decalactone has characteristic peach and apricot flavors and its low detection threshold of 0.088 ppm in the water (Siek *et al*., 1971). Moreover, a selection of strains of different species of Candida able to β-oxidize castor oil hydrolysate (containing ricinoleic acid) to produce γ -decalactone was patented (Boog *et al*., 1990). Most

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industrial processes use the bioconversion of ricinoleic acid by yeasts to produce γ -decalactone (Aguedo *et al*., 2004; Schrader *et al*., 2004). The yeast *Yarrowia lipolytica* is able to transform a hydroxylated C18 fatty acid into γ -decalactone and is also able to degrade the produced lactone (Groguenin *et al*., 2004).

In *Yarrowia lipolytica*, acyl-CoA oxidases are encoded by five genes (POX1 through POX5). An investigation of the function of the Aox isozymes demonstrated a chain length specificity for Aox2p (long chain) and Aox3p (short chain). Aox2p and Aox3p together account for 70 to 80% of global Aox activity Pagot *et al*., (1998) disrupted POX1 (encoding Aox1p) and observed higher levels of β-oxidation and higher Aox activities but lower levels of lactone production, thereby demonstrating that Aox plays a fundamental role in lactone production. So far, no technological applications have resulted from the research of yeast acyl-CoA oxidases apart from that of Picataggio *et al*., (1992), who blocked β -oxidation by sequential disruption of Aox genes in *Candida tropicalis* to redirect fatty acids to the β -oxidation pathway, leading to the production of long-chain dicarboxylic acids. Acyl-CoA oxidases catalyse the first reaction of the β -oxidation cycle and they are generally considered as the ratelimiting enzyme of sequence (Wache *et al*., 1998; Wang *et al*., 1998).

The recent genomic exploration of *Y. lipolytica* highlighted the presence of 16 genes coding for lipase (Patrick *et al*., 2011). Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme as the quantities produced by wild strains are usually too low (Haq *et al*., 2009). The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection of microorganisms (Bapiraju *et al*., 2004; khattab and Bazaraa 2005). UV and NTG (Nmethyl- N'-nitro-N-nitroso guanidine) was further reported as effective mutagenic agents for strain improvement of Rhizopus sp. BTS-24 for productivity of biomedically important enzyme lipase. Gromada and Fiedurek (1997) have also developed strain improvement by induced mutagenesis with rational selection procedures for an efficient screening of the mutants. Random mutagenesis by ultraviolet (UV) rays and/or chemical mutagens followed by fermentation screening has been reported as an effective way to improve the Lipase productivity of *Yarrowia lipolytica* (Destain *et al*., 1997; Mansour *et al*., 2002; Fickers *et al*., 2003). Furthermore, the RAPD technique by PCR has been shown to be extremely useful in evaluation of genetic variability of microbial strains. Several authors cite the RAPD as ideal in the study of genomic polymorphism. This method has been used to compare intra- and interspecific differences in bacteria. The purified DNA or the cell extracts cultivated in agar can be used (Williams *et al*., 1990; Ikeh 2003).

The purpose of this work is to improve the biotransformation of castor oil into gamma-decalactone using lipase produced by genetically improved strains of *Y. lipolytica* as UV-mutants and evaluated the genetic variability of these mutant strains by RAPD analysis.

MATERIALS AND METHODS

Microorganism:

Y. lipolytica ATCC 20226 strain was obtained from the culture collection of the ATCC, USA.

Growth Conditions:

Y. lipolytica strain preserved at 4°C on YPD-agar medium (Amaral *et al*., 2007). For cultivation, the strain was grown in 250 mL flasks contained 50mL YPD or production media and incubated at a rotary shaker adjusted to 200 rpm at 30°C for two days.

Production Media:

The composition of the medium for lipase and γ-decalactone production (Adelaide *et al*., 2012) with some modification was: castor oil 20 g/L, yeast extract 3 g/L, peptone 3 g/L, casein 3 g/L, Tween 1 g/L, yeast nitrogen base 6.7 g/L. Initial pH of the media was adjusted to be 6.

UV-Mutagenesis:

Five ml of *Y. lipolytica* ATCC 20226 cells was irradiated with Philips TUV-30-W-254 nm Lamp (Philips, Holland) for 4, 8 and 12 min at a distance of 20 cm. The irradiated cells were kept (2 hr) in dark to avoid photoreactivation repair. Suspension was then diluted and spread onto the surface of SG-CAA medium (Liu *et al*., 2010) contained tributyrin (0.5% v/v) for selection of high-producing lipase colonies. Plates were then incubated for 3 days at 30°C. The growing colonies with high clearing zones were transferred on slants for further studies. At this stage, a reducing in viability was about 90%. Mutants with high clearing zones were then recultured on the same medium plates for 3 days at 30°C.

Extracellular Lipase Activity Determination:

Lipase activity was assayed against p-nitrophenyl-palmitate (pNP-P) as substrate. Extracellular fluid containing enzyme was incubated with pNP-P (8.0 mM, dissolved in isopropanol), 0.05M sodium phosphate buffer (pH 8.0) and Triton X- 100 (0.4%, w/v) in a final volume of 1ml at 35 ◦C for 30 min. The developed

color was measured at 410 nm against blank did not contain enzyme solution. One unit of lipase activity is defined as the release of 1µmol of pNP per min. The standard was carried out.

Lactone Extraction and Analyses:

For the quantification of γ -decalactone, 2-mL medium samples were removed and their pH was adjusted to be 2.0 with HCL. The extraction of the aromatic compound was performed with 2 mL diethyl ether by 60 gentle shakes, after addition of γ -decalactone, as internal standard. The petroleum ether was recovered and dehydrated by anhydrous Na_2SO_4. The concentration of γ-decalactone was determined by Perkin Elmer Autosystem equipped with flame ionization detector (FID) (gas chromatography) GC as described by Groguenin *et al*. (2004) using He as a carrier gas at a linear flow rate of 1 ml/min. The split injector (split ratio, 10:1) and the flame ionization detector temperature were set to 250 ^{0}C. The initial oven temperature was programmed to increase from 60 ^{0}C to 150 ^{0}C at a rate of 10 ^{0}C/min holding for 1 min and then to 250 ^{0}C at a rate of 15 ^{0}C/min, holding for 10 min. GC experiments were repeated for three times. The standard curve of γ-decalactone was made by plotting a serial of concentration of standard γ-decalactone solutions against peak areas appeared on GC diagram; consequently the quantification of γ-decalactone was gotten on the standard curve.

Isolation of Total DNA from Mutant Strains:

Total DNA was isolated according to Khattab (1997) with using (10 mg/ml) of Snail enzyme. 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).

Molecular Analysis of Some Superior Mutants by PCR:

PCR-GOLD Master-Mix Beads (BIORON, Germany, Cat. No. 10020-96) 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. Two different primers were used in the present study. The first primer sequence was 5'-GTA GAC CCG T-3'. The second primer sequence was 5'- GTC CAC ACG G-3'. The third primer sequence was 5'- GAC GGA TCA G-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 at 35^{o}C 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 1.5% 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 (Pharmacia Biotech.) and the separated bands were stained with0.5 µg/ml ethidium bromide and photographed using Gel Documentation System with UV-Transeliminator.

RESULTS AND DISCUSSION

UV Mutagenesis and Lipase Production:

This study was mainly planned aiming to improve of the gene responsible for lipase production through UV treatment of *Y. lipolytica* with different doses. Screening of 300 UV-treated colonies, only 72 were characterized as high lipase producer mutants (Tables 1, 2 and 3). These mutants were tested based on both clear zone (C) and growth zone (G) of some superior *Y. lipolytica* mutants to evaluate their lipase productivity (Fig. 1). The results presented in Table (1) showed that the exposure to UV-light for 4min. enhanced the lipase relative production (C/G) up to 65.94 percent for the mutant 22/4 compared to the original strain. The mutants No. 14/4 and 19/4 which exceeded by 44.93 % and 55.07 %, respectively, than the original strain. On the other hand, 4 mutants exhibited lower productivity than the parental strain.

Data in Table (2) clearly showed that the mutants exhibited more productive than the original parent except two mutants (7/8 and 10/8) exhibited lower lipase productivity. Furthermore, the highest producer mutants i.e., 12/8 and 22/8 exceeded their original strain with 76.09 and 65.94 %.

Data in Table (3) clearly showed that all induced mutants were high productive than the original strain. Furthermore, the highest record of the lipase production was 3.17 based on the lipase relative production (C/G), with 129.71% more than the original strain, which obtained from the mutant No. 10/12.

Data in Table (4) present the lipase productivity measured as units/ml of some superior *Y. lipolytica* mutants obtained after UV-mutagenesis. The obtained results showed that the same trend to those obtained after lipase measured as relative production (C/G). Results clearly showed that all mutants were more productive than the original strain (W.T). Furthermore, the highest record of the lipase production was125.25 U/ml, with 209.26% over than the wild type strain, and obtained from the mutant No. 10/12. Meanwhile, the rest mutants produced lipase higher than the wild type strain but lower than the superior mutant No. 10/12.

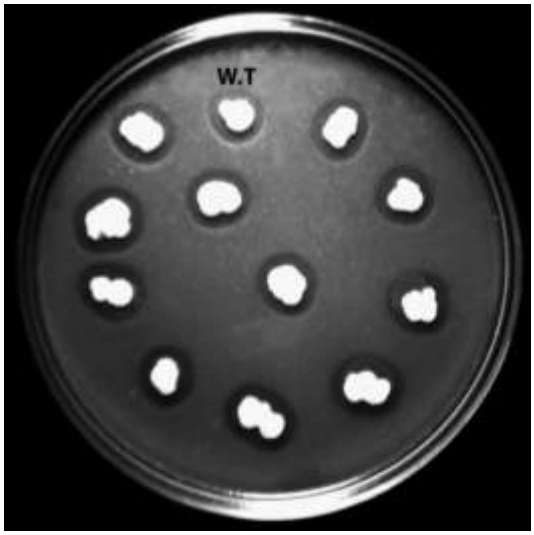

Fig. 1: Photograph of some superior *Y. lipolytica* mutants for lipase production in comparison with their original strain (W.T).

Table 1: Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* mutants obtained after 4 min UV-treatment.

Mutant No.	G	C	C/G	C/G % to W.T
W.T	8	11	1.38	100.00
1/4	7	12	1.71	123.91
2/4	9	11	1.22	88.41
3/4	8	13	1.62	117.39
4/4	8	12	1.50	108.70
5/4	7	11	1.57	113.77
6/4	8	14	1.75	126.81
7/4	9	12	1.33	96.38
8/4	9	14	1.56	113.04
9/4	10	15	1.50	108.70
10/4	7	12	1.71	123.91
11/4	8	14	1.75	126.81
12/4	10	15	1.50	108.70
13/4	10	16	1.60	115.94
14/4	7	14	2.00	144.93
15/4	11	13	1.18	85.51
16/4	8	14	1.75	126.81
17/4	8	14	1.75	126.81
18/4	9	16	1.78	128.99
19/4	7	15	2.14	155.07
20/4	10	14	1.40	101.45
21/4	10	13	1.30	94.20
22/4	7	16	2.29	165.94
23/4	8	14	1.75	126.81
24/4	9	13	1.44	104.35

Also, data in Table (3) clearly showed that all induced mutants were more productive than the original strain. Furthermore, the highest record of the lipase production was 3.17 based on the lipase relative production (C/G), with 129.71% more than the wild type strain, which obtained from the mutant No. 10/12.

Data in Table (4) presents the lipase productivity measured as units/ml of some superior *Y. lipolytica* mutants obtained after UV-mutagenesis. The obtained results showed that the same trend to those obtained after lipase measured as relative production (C/G). Results clearly showed that all mutants were more productive than the original strain (W.T). Furthermore, the highest record of the lipase production was125.25 U/ml, with 209.26% over than the original strain, which obtained from the mutant No. 10/12. Meanwhile, the rest mutants produced lipase higher than the wild type strain but lower than the superior mutant No. 10/12.

From the above results, it appeared that this mutant selection was a good strategy for improving the productivity of *Yarrowia lipolytica* lipase and the selection of the highest lipolytic zone with tributyrin (0.5% v/v) can be successfully used as a selection procedure to improve the lipase synthetic capacity of the original strain. Mutagenesis has been used as a breeding method to select superior lipase mutants of *Yarrowia lipolytica* (Fickers *et al*., 2003).

These results are in agreement with those of Destain *et al*., 1997. Who isolated Mutants increased capacities of lipase secretion from CBS6303 by chemical mutagenesis using N-methyl-N-nitro-N-nitrosoguanidine (NTG). NTG is previously also reported as an effective mutagen for strain improvement of *Rhizopus* spp for enhanced lipase activity (Bapiraju *et al*., 2004). The selected mutant was therefore assigned the code IIB-63NTG-7 and used for further studies in parallel with wild strain. Moreover, Mansour *et al*., 2002 treated *Y. lipolytica* by UV-mutagenesis to obtain mutants with high ability to hydrolyze olive oil. The promising mutant No.15 was used in protoplast fusion with the parental strain to obtain high lipase productive fusants which able to lactonization of 4-hydroxydodecanoic acid to form γ -dodecalactone.

Table 2: Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* mutants obtained after 8 min UV-treatment.

Mutant No.	G	C	C/G	C/G % to W.T
W.T	8	11	1.38	100.00
1/8	8	12	1.50	108.70
2/8	7	13	1.86	134.78
3/8	8	13	1.62	117.39
4/8	7	12	1.71	123.91
5/8	7	14	2.00	144.93
6/8	10	14	1.40	101.45
7/8	9	12	1.33	96.38
8/8	8	14	1.75	126.81
9/8	9	15	1.67	121.01
10/8	10	12	1.20	86.96
11/8	8	14	1.75	126.81
12/8	7	17	2.43	176.09
13/8	10	16	1.60	115.94
14/8	7	13	1.86	134.78
15/8	8	12	1.50	108.70
16/8	8	14	1.75	126.81
17/8	7	14	2.00	144.93
18/8	8	16	2.00	144.93
19/8	7	15	2.14	155.07
20/8	10	16	1.60	115.94
21/8	6	13	2.17	157.25
22/8	7	16	2.29	165.94
23/8	9	14	1.56	113.04
24/8	8	13	1.62	117.39

Table 3: Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* mutants obtained after 12 min UV-treatment.

Mutant No.	G	C	C/G	C/G % to W.T
W.T	8	11	1.38	100.00
1/12	6	14	2.33	168.84
2/12	9	15	1.67	121.01
3/12	7	13	1.86	134.78
4/12	9	20	2.22	160.87
5/12	7	12	1.71	123.91
6/12	6	12	2.00	144.93
7/12	8	12	1.50	108.70
8/12	7	18	2.57	186.23
9/12	10	17	1.70	123.19
10/12	6	19	3.17	229.71
11/12	8	17	2.13	154.35
12/12	10	15	1.50	108.70
13/12	8	16	2.00	144.93
14/12	6	14	2.33	168.84
15/12	8	12	1.50	108.70
16/12	8	16	2.00	144.93
17/12	8	16	2.00	144.93
18/12	10	17	1.70	123.19
19/12	10	15	1.50	108.70
20/12	8	14	1.75	126.81
21/12	9	16	1.78	128.99
22/12	6	14	2.33	168.84
23/12	8	18	2.25	163.04
24/12	8	17	2.13	154.35

Table 4: Lipase production measured as units/ml of some superior *Y. lipolytica* mutants obtained after UV-treatment.

Mutant No.	Lipase U/ml	% to W.T	Mutant No.	Lipase U/ml	% to W.T
W.T	40.50	100.00	22/8	98.50	243.21
14/4	85.24	210.47	1/12	101.44	250.47
19/4	87.50	216.05	4/12	93.25	230.25
22/4	98.44	243.06	8/12	111.55	275.43
5/8	84.25	208.02	10/12	125.25	309.26
12/8	105.23	259.83	11/12	92.54	228.49
17/8	82.65	204.07	14/12	95.48	235.75
18/8	81.87	202.15	22/12	92.54	228.49
19/8	86.45	213.46	23/12	93.44	230.71
21/8	87.90	217.04	24/12	85.52	211.16

γ-Decalactone Productivity of Some Superior Y. lipolytica Mutants:

The production of γ -decalactone by some superior mutants obtained after UV-treatments is shown in Table 5. The wild type produced γ -decalactone concentration reached 17.6 mg/L. In contrast, the superior mutant No. 10/12 attained 89.17 mg/l, with 406.65% more than the wild type strain. The mutants 8/12 exceeded than original strain by 359.03%. Meanwhile, the rest mutants produced γ -decalactone higher than the wild type strain but lower than the superior mutants No. 10/12 and No. 8/12. In general, data also indicated very highly positive correlation between the amount of lipase produced and the amount of γ –decalactone produced by the superior UV-mutants.

These data support previous findings that *Y. lipolytica* possess a family of 6 genes (POX1 to POX6) coding for acyl-CoA oxidases (Aox1p to 6p) with some being chain-length selective (Wang *et al*., 1998). Deleting genes coding for short-chain selective acyl-CoA oxidases enabled to increase the production of γ-decalactone (Wache *et al*. 2000) and decrease the one of other lactones (Wache *et al*. 2001). Following these observations, an attempt to block β-oxidation on short-chains was carried out and it resulted in a 10-fold increase in the yields (Wache *et al*. 2002; Groguenin *et al*. 2004). Some acyl-CoA oxidase evolution experiments were carried out by shuffling POX2 and POX3 (encoding the long-chain and short-chain specific enzyme, respectively) or mutating this latter enzyme. However, Gatfield *et al*. (1993) found that the wild type accumulated hydroxylated and unsaturated lactones as γ-decalactone was degraded. However, a strain lacking the short-chain Aox (Aox3) behaved similarly, except that this mutant accumulated the high amount of γ-decalactone.

Table 5: Production of γ-decalactone by the highly *Y. lipolytica* mutants obtained after UV-mutagenesis.

Mutant No.	γ-decalactone (mg/L)	% to W.T	Mutant No.	γ-decalactone (mg/L)	% to W.T
W.T	17.60	100.00	22/8	60.39	343.13
14/4	48.82	277.39	1/12	67.11	381.31
19/4	51.88	294.77	4/12	54.00	306.82
22/4	59.07	335.63	8/12	80.79	459.03
5/8	45.27	257.22	10/12	89.17	506.65
12/8	69.43	394.49	11/12	52.94	300.80
17/8	44.43	252.44	14/12	55.18	313.52
18/8	42.79	243.13	22/12	40.51	230.17
19/8	50.11	284.72	23/12	54.84	311.59
21/8	52.38	297.61	24/12	49.17	279.38

Molecular Analysis of Some Superior Y. lipolytica Mutants:

To evaluate the genetic effect of UV-mutagenesis on the DNA nucleotide sequence of the obtained mutants compared to the original strain. Three random primers obtained from Operon Technologies were used to identify the genetic variability among the five superior mutants. All these primers were successfully amplified specific fragments of the genomic DNA. The RAPD technique was used in order to test if genetic markers could be correlated with the production of lipase and γ-decalactone.

The application of primer No.1 (Fig. 2) with the genomic DNA of the wild type strain and five superior mutants clearly noticed that ten amplified bands were occurred when DNA of the wild type strain (lane 2) was used as a template. Also, the same ten amplified bands were occurred when DNA of the four mutants i.e., No. 22/4, 1/12, 8/12 and 10/12 (lanes 3, 5, 6 and 7) were used as a template. Furthermore, in the lane 4 of the mutant No. 22/8 obtained after UV-mutagenesis for 8 min., a new distinct band with size of 600 bp was detected.

On the other hand, the application of primer No.2 (Fig. 3) with the genomic DNA of the wild type strain and five superior mutants clearly noticed that twelve amplified bands were occurred when DNA of the wild type strain (lane 2) was used as a template. The same twelve amplified bands were occurred when DNA of the five mutants No. 22/4, 22/8, 1/12, 8/12 and 10/12 (lanes 3 to 7) were used as a template. Moreover, the amplification reactions with the primer No.3 (Fig. 4) generated a total of 44 distinct fragments, 6(13.64%) of them being polymorphic as shown in lanes 3, 5, 6 and 7.

The above differences in RAPD profiles confirmed the evidence of changes in *Y. lipolytica* genome after UV-mutagenesis. Furthermore, some of these differences based on RAPD technique, could be used as genetic markers for screening of the new induced mutants after UV-mutagenesis.

The obtained results are in agreement with those reported by Schlick *et al*., (1994) who demonstrated that some differences and similarities in RAPD profile between mutants in *Trichoderma harzianum* obtained after gamma radiation. Moreover, Barcelos *et al*., (2011) reported that, RAPD markers were useful for detecting genetic variability among isolates of *Colletotrichum lindemuthianum*. Khattab and Abd El-Salam (2012) noticed some differences in mutant strains in comparison with the original strain. These differences in RAPD profiles confirmed the evidence of genetic variations of mutants and *Mucor racemosus* genome after UV-mutagenesis.

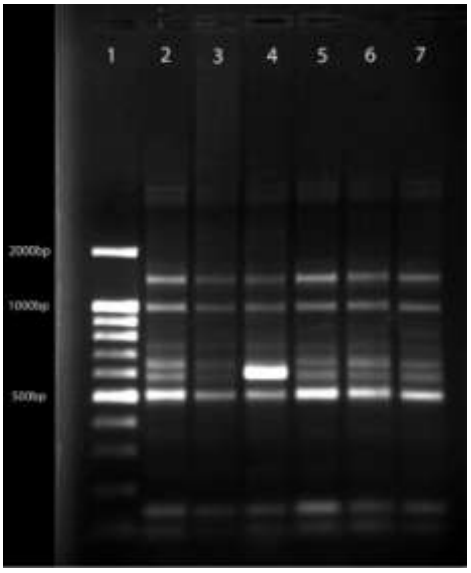

Fig. 2: Photograph of DNA amplified banding patterns based on RAPD for five superior mutants against wild type strain (lane 2) and 100 bp ladder DNA marker (lane 1) using primer No.1. Mutants sequence as follows: (lane 3 to 7), 22/4, 22/8, 1/12, 8/12 and 10/12.

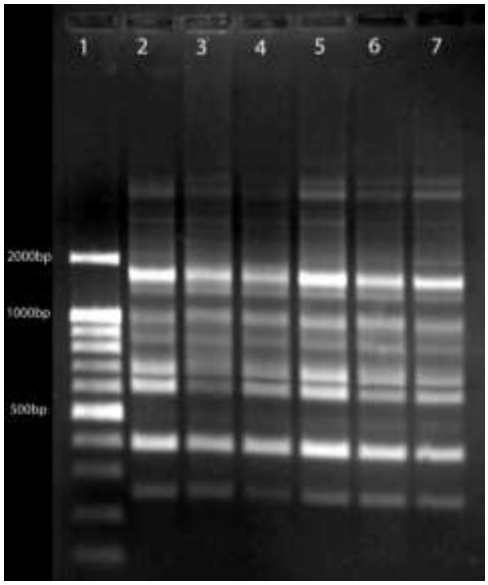

Fig. 3: Photograph of DNA amplified banding patterns based on RAPD for five superior mutants against wild type strain (lane 2) and 100 bp ladder DNA marker (lane 1) using primer No.2. Mutants sequence as follows: (lane 3 to 7), 22/4, 22/8, 1/12, 8/12 and 10/12.

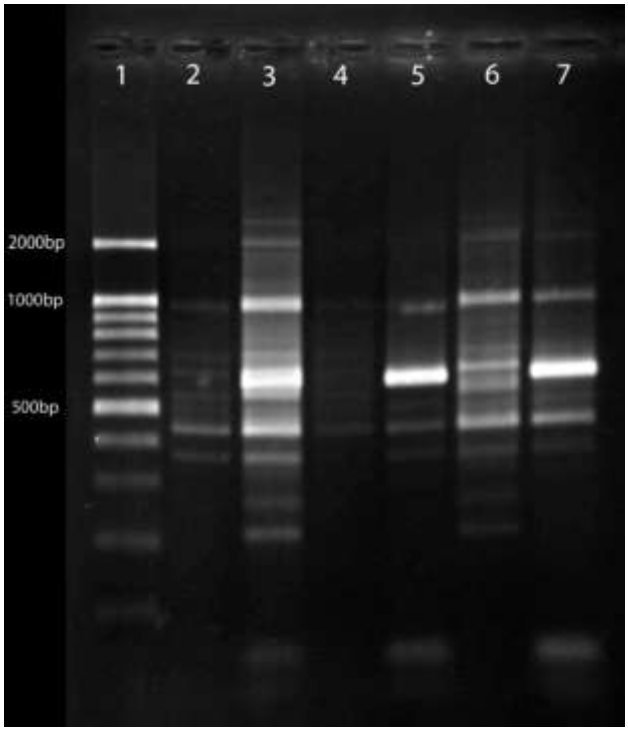

Fig. 4: Photograph of DNA amplified banding patterns based on RAPD for five superior mutants against wild type strain (lane 2) and 100 bp ladder DNA marker (lane 1) using primer No.3. Mutants sequence as follows: (lane 3 to 7), 22/4, 22/8, 1/12, 8/12 and 10/12.

In conclusion, our study has revealed that an increase in lipase production by *Y. lipolytica* mutants after UV-treatment was obtained and this was related to the production of γ-decalactone. Furthermore, the application of RAPD technique on some selected superior mutants in comparison with the wild type lead to detect of some differences in RAPD patterns which used as evidence of genetic variations of *Y. lipolytica* mutants after UV-treatment and used as tool to draw the DNA fingerprint of some promising mutants at the commercial field.

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