Research article

Enhancing γ-decalactone production by protoplast fusion of *Yarrowia lipolytica*

Gamil Ibrahim,[1*] Abd-Elnaser Khattab,[2] Hamdy Shaaban,[1] Magda Abd El-Mageed,[1] FatmaTalkhan,[2] Fouad Osman[1]

[1] Flavours & Aroma Chemistry Department, National Research Center, Cairo, Egypt
[2] Genetics and Cytology Department, National Research Center, Dokki, Cairo, Egypt

*gamilemad2000@gmail.com

Abstract: *γ-Decalactone is an aroma compound with a pleasant peachy odour, it can be produced by Yarrowia lipolytica, but the yield is commonly poor. Therefore, the present study aimed to use protoplast fusion performed between different superior mutants of Y. lipolytica to improve γ-decalactone production. Data showed that 45 fusants obtained from all three crosses, the cross 1 enhanced the lipase relative production (C/G) up to 106.24% for the fusant C1/9 over the original strain. Furthermore, the highest record of the lipase production was 9.50% based on the lipase relative production (C/G), with 227.82% more than the original strain, which obtained from the fusant No. C2/3 (cross2). Moreover, the highest record of the lipase production was 10 based on the lipase relative production (C/G), with 239.81% more than the wild type strain, which obtained from the fusants C3/2 and C3/14 (cross3). Finally, the highest record of the γ-decalactone production was 108.78 mg/L with 618.07% more than the wild type strain, which obtained from the fusant C3/14.*

Keywords: *Yarrowia lipolytica, protoplast fusion, γ-decalactone.*

Introduction

Lactones are widely distributed in foods and beverages. They have been found in the aromas of more than 120 foodstuffs, which explains their importance in the aroma industry. An increasing demand for natural products has resulted in the use of biotechnological processes for the production of these lactones. This has led to numerous patents being taken out, and nowadays the biotechnologically produced lactone family is mainly represented by γ-decalactone, but also to a smaller extent by γ-dodecalactone and γ-octalactone [1]. γ-Decalactone has an oily-peachy, extraordinarily tenacious odour and a very powerful, creamy-fruity, peach-like taste in concentrations below 5 ppm [2]. It is used in the formulation of fruit aromas such as strawberry, apricot and peach, but is also present in many fermented products such as bread and whiskies [3].

With its tempting aroma and low odour threshold (0.088 ppm in water) [4], γ-decalactone is recognized internationally as a safe food additive [5] and obtained the characteristics of the universal application in the fragrance industry [6]. The discovery of its accumulation during ricinoleate catabolism stimulated studies on the processes of production reviewed in [1,7]. As a consequence of these studies, γ-decalactone is now one of the aroma compounds most produced through biotechnology. As a second consequence, its price, which was close to $12 000/kg in 1986, has fallen to under $ 100/kg [8]. However, the demand is very high and the low price stimulates research to improve the biotransformation.

γ-Decalactone can be produced from methyl ricinoleate, the major component of esterified castor oil, through a degradation pathway that is present in several yeast species, the most efficient species is *Yarrowia lipolytica* [7]. It is considered as a model organism for the metabolism of hydrophobic compounds and therefore, it has been extensively studied [9].

Yarrowia lipolytica has been used in the industrial production of γ-decalactone, but the yield of this biotransformation is commonly poor**.** Apart from β-oxidation activity, another factor influencing the yields is the toxicity of the lactone. The mechanisms involved in this toxicity have been characterized showing that the carbon-lateral chain of the lactone interacts with membranes, increasing their fluidity and decreasing their integrity [10].

Current literature did not reveal any use of protoplast fusion techniques to improve γ-decalactone production by *Y. lipolytica*. Therefore, this study was initiated to take advantage of the protoplast fusion technique in *Y. lipolytica* to obtain γ-decalactone overproducing strains.

Experimental

Materials

The original strain of *Y. lipolytica* ATCC 20226 was obtained from the culture collection of the ATCC, USA and the superior mutants (Nos., 4/12, 12/8, 8/12 and 10/12) of *Y. lipolytica* were obtained from the previous study [11].

Methods

Growth Conditions

Y. lipolytica strains conserved at 4^{o}C on YPD-agar media [12]. For cultivation, the strains were grown at 30^{o}C and 200 rpm in 50 mL YPD or production media in 250 mL flasks.

Production Medium

The composition of the medium for lipase and γ-decalactone production as mentioned in [13] with some modification was as following: castor oil 20 g/L, yeast extract 3 g/L, peptone 3 g/L, casein 3 g/L, yeast nitrogen base 3 g/L. The media was adjusted at initial to be at pH 6.0.

Protoplast formation

The different superior mutants were grown in 50 mL of protoplasting medium as described by [14] and incubated on a rotary shaking incubator (150 rpm) at 30°C for 18 hrs. Cells were collected by centrifugation and washed twice with sterile distilled water. The washed cells were resuspended in the pre-treatment solution and the suspension was incubated for 20 min at 32°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°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.

Porotoplast fusion

Protoplast fusion could be carried out between selected isolates which differ in either their resistance to heavy metals or lipase production levels as follow: protoplasts from different cultures were mixed, centrifuged at 2500 rpm for 5 min and the supernatant was removed. Two mL of polyethelene glycol (PEG) solution were added and mixed gently with protoplasts [15]. The mixture of protoplasts was incubated up to 30 min at 30°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 surface of (SG-CAA) media [16] containing tributyrin (0.5% v/v) for selection of high-producing lipase colonies and 0.65 M KCl for maintain the protoplast cells without burst. Plates were then incubated (3 days, 30°C) and the growing colonies (fusants) with high clearing zones were transferred on slants for further studies. Fusants with high clearing zones were then retested on the same plates and selected of the superior fusants for lipase and γ-decalactone production.

Extraction and analysis of γ-decalactone

To quantify lactones during the methyl ricinoleate biotransformation, 1.5-mL samples were taken and poured in 4-mL glass. Then 10 µL 36% HCl was added to stop metabolism and to reach complete lactonization of 4-hydroxy acids. An internal standard (undecanoic γ-lactone, Sigma-Aldrich), 10 µL solubilized in absolute ethanol, was added to reach a final concentration of 100 mg/L. The sample was then extracted with 1.5 mL diethyl ether inverting the vial every second for 90 s. After 15 min, the ether phase was extracted and analyzed by gas chromatography (Chromatograph Hewlett-Packard HP5890, Agilent technology series; Lyons, France), with a DB-5 capillary column (Agilent, 60 m × 320 µm × 0.25 µm) with He as a carrier gas at a linear flow rate of 1 mL/min. The split injector (split ratio, 1:10) temperature was set to 250°C and the flame ionization detector, to 300°C. The oven temperature increased from 60°C to 145°C at 5°C/min, and finally at 2°C/min to reached 215°C [17].

Results and Discussion

Response of superior mutants to different heavy metals

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

Data presented in Table 1 showed the resistance or sensitivity to benomyl (B), cycloheximide (C), Griseofulvin (G), nystatin (N), miconazole (M) and some of heavy metals: selenium (Se), barium (Ba), mercury (Hg), arsenate (As), cadmium (Cd), cobalt (Co) and cesium (Cs). Results showed that, although the original strain was resistant to all antifungal agents, most of the selected mutants exhibited the same antifungal response except the mutants Nos., 4/12, 12/8 and 8/12 proved to be sensitive to N. On the other hand, the original strain and all mutants were resistant to Ba and Cs. However, most of the selected mutants exhibited different response to the other heavy metals.

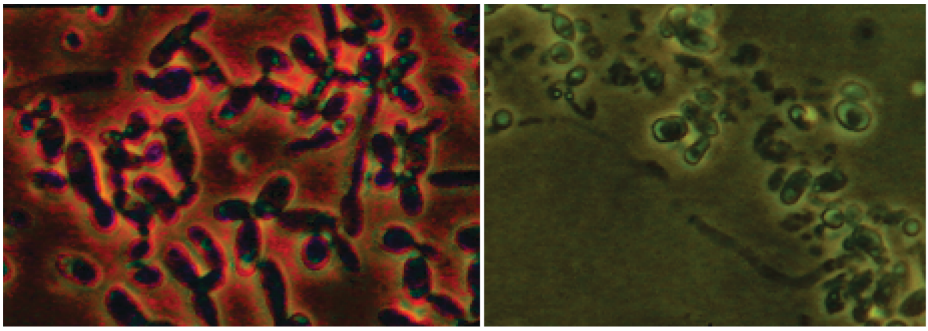
Table 1. Antifungal and heavy metals response of the original strain (W.T) and the superior mutants selected for protoplast fusion

Strain No.	G	N	C	B	Se	Ba	Hg	As	Cd	Co	Cs
W.T	+	+	+	+	+	+	−	−	−	−	+
4/12	+	–	+	+	+	+	–	–	–	–	+
12/8	+	–	+	+	–	+	+	–	+	+	+
8/12	+	–	+	+	–	+	+	+	–	+	+
10/12	+	+	+	+	+	+	+	+	–	+	+

However, three mutants (12/8, 8/12 and 10/12) were selected and introduced subsequently into protoplasting, fusion and regeneration. Two mutants, i.e., 12/8 and 8/12 were used as parental strains for cross1 where As and Cd were used as selective markers. Mutants Nos., 12/8 and 4/12 were used for cross 2 where Se and Cd were used as selective markers. Finally, 4/12 and 12/8 were used for cross 3 where Se and As were used as selective markers.

Protoplast fusion

Among the wide variety of strain improvement protocols, protoplast fusion seems to be an efficient way to induce genetic recombination. This method has also been proved to be valuable in the development of new industrial yeast strains. However, under the conditions mentioned under materials and methods, protoplasts can be isolated as shown in Figure (1/B). 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 [18].



A **B**

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

Anyhow, 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°C for further genetic analysis.

Protoplast fusion and lipase production

This research was mainly planned aiming the combination and improvement of the genes responsible for lipase and γ-decalactone production through protoplast fusion of *Y. lipolytica*. After different crosses of protoplast fusion between superior UV-mutants, the obtained fusants were tested for lipase activities on SG-CAA medium plates containing tributyrin (0.5%, v/v) and screening about 150 fusants colonies, only 45 were characterized as high lipase producer mutants (Tables 2, 3 and 4). These fusants were tested based on both clear zone (C) and growth zone (G) of some superior *Y. lipolytica* fusants to evaluate their lipase productivity (Figure 2).

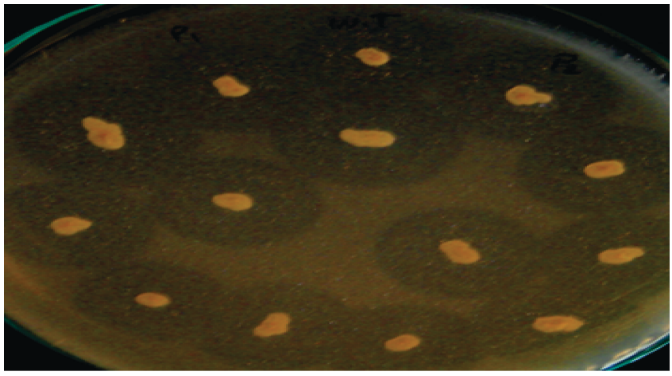


Figure 2. Photograph of some superior *Yarrowia lipolytica* fusants for lipase production in comparison with their original strain

The results presented in Table (2) showed that the cross1 enhanced the lipase relative production (C/G) up to 106.24% for the fusant C1/9 over the original strain. The next highest producer fusants were C1/14 and C1/4 which exceeded their original strain by 96.64 % and 91.85 %, respectively.

On the other hand, the lipase relative production (C/G) enhanced up to 34.38% for the fusant C1/9 over the high first parent (P1).

Table 2. Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* fusants obtained after cross 1

Mutant No.	C	G	C/G	C/G % to W.T	C/G % to P1
W.T	25	6	4.17	100.00	65.16
P1-8/12	32	5	6.40	153.48	100.00
P2-12/8	30	5	6.00	143.88	93.75
C1/1	38	5	7.60	182.25	118.75
C1/2	41	7	5.86	140.53	91.56
C1/3	38	5	7.60	182.25	118.75
C1/4	40	5	8.00	191.85	125.00
C1/5	38	7	5.43	130.22	84.84
C1/6	38	6	6.33	151.80	98.91
C1/7	41	7	5.86	140.53	91.56
C1/8	37	5	7.40	177.46	115.63
C1/9	43	5	8.60	206.24	134.38
C1/10	38	7	5.43	130.22	84.84
C1/11	33	7	4.71	112.95	73.59
C1/12	40	6	6.67	159.95	104.22
C1/13	35	6	5.83	139.81	91.09
C1/14	41	5	8.20	196.64	128.13
C1/15	38	5	7.60	182.25	118.75

Also, data in Table 3 clearly showed that all induced fusants were more productive than the original strain. Furthermore, the highest record of the lipase production was 9.50% based on the lipase relative production (C/G), with 227.82% more than the original strain, which obtained from the fusant No. C2/3. The next highest producer fusants were C2/10 and C2/4 which exceeded their original strain by 96.64 % and 91.85 %, respectively. On the other hand, the lipase relative production (C/G) enhanced up to 34.38% for the fusant C1/9 over the high first parent (P1).

On the other hand, 15 selected fusants obtained from cross 3 in the presence of Se and As. Results in Table 4 showed that all of the fusants exhibited more lipase production than its wild type (W.T). Increasing lipase productivity is ranging from 127.82 to 239.81% higher than the wild type. The highest record of the lipase production was 10 based on the lipase relative production (C/G), with 239.81% more than the wild type strain, which obtained from the fusants C3/2 and C3/14. However, the tested fusants from cross 3 proved to be higher effective for increasing lipase production than fusants obtained from cross 2.

Table 3. Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* fusants obtained after cross 2

Mutant No.	C	G	C/G	C/G % to W.T	C/G % to P1
W.T	25	6	4.17	100.00	57.52
P1-4/12	29	4	7.25	173.86	100.00
P2-12/8	30	5	6.00	143.88	82.76
C2/1	35	5	7.00	167.87	96.55
C2/2	32	5	6.40	153.48	88.28
C2/3	38	4	9.50	227.82	131.03
C2/4	41	5	8.20	196.64	113.10
C2/5	36	6	6.00	143.88	82.76
C2/6	40	6	6.67	159.95	92.00
C2/7	39	5	7.80	187.05	107.59
C2/8	37	5	7.40	177.46	102.07
C2/9	32	4	8.00	191.85	110.34
C2/10	34	4	8.50	203.84	117.24
C2/11	38	5	7.60	182.25	104.83
C2/12	33	6	5.50	131.89	75.86
C2/13	35	7	5.00	119.90	68.97
C2/14	40	7	5.71	136.93	78.76
C2/15	38	5	7.60	182.25	104.83

Table 4. Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* fusants obtained after cross 3.

Mutant No.	C	G	C/G	C/G % to W.T	C/G % to P1
W.T	25	6	4.17	100.00	57.52
P1-4/12	29	4	7.25	173.86	100.00
P1-8/12	32	5	6.40	153.48	88.28
C3/1	33	5	6.60	158.27	91.03
C3/2	40	4	10.00	239.81	137.93
C3/3	32	6	5.33	127.82	73.52
C3/4	38	7	5.43	130.22	74.90
C3/5	40	5	8.00	191.85	110.34
C3/6	37	5	7.40	177.46	102.07
C3/7	38	5	7.60	182.25	104.83
C3/8	36	4	9.00	215.83	124.14
C3/9	40	6	6.67	159.95	92.00
C3/10	31	5	6.20	148.68	85.52
C3/11	38	4	9.50	227.82	131.03
C3/12	39	6	6.50	155.88	89.66
C3/13	40	6	6.67	159.95	92.00
C3/14	40	4	10.00	239.81	137.93
C3/15	37	5	7.40	177.46	102.07

In general, protoplast fusion between higher lipase production mutants proved to be effective to achieve superior lipase production fusants [19]. Therefore, protoplast fusions have been used successfully to enhancement the lipase production. The recombinants strains can be obtained by this technique [20-22].

From the above results, it appeared that the protoplast fusion was a good strategy for improving the lipase productivity of *Y. lipolytica* 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. These results are in accordance with those of Mansour et al. [23], they treated *Y. lipolytica* by UV-mutagenesis to obtain mutants with high ability to hydrolyze olive oil. Some induced mutants over yielded their original strain in oil hydrolysis. 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.

Protoplast fusion and γ-decalactone production

Data in Table 5 presents the γ-decalactone productivities of some superior *Y. lipolytica* fusants obtained after different crosses of protoplast fusion. Results clearly showed that all fusants were more productive than the wild type (W.T). Furthermore, the highest record of the γ-decalactone production was 108.78 mg/ L with 618.07% more than the wild type strain, which obtained from the fusant C3/14. The next highest producer fusants were C1/9 and C3/7 which exceeded their original strain by 595.17% and 467.84%, respectively. Meanwhile, the rest fusants produced γ-decalactone higher than the wild type strain but lower than the superior fusant C3/14.

Table 5. γ-Decalactone production of some superior fusants obtained after different protoplast fusion crosses

Parents & fusants	γ-decalactone (mg/L)	% to W.T	Parents & fusants	γ-decalactone (mg/L)	% to W.T
W.T	17.60	100.00	C2/4	37.24	211.59
C1			C2/7	59.09	335.74
P1-8/12	80.79	459.03	C2/9	59.88	340.23
P2-12/8	69.43	394.49	C2/10	51.75	294.03
C1/1	17.76	100.90	C2/11	54.06	307.16
C1/3	19.34	109.87	C3		
C1/4	17.68	100.45	P1-4/12	54.00	306.82
C1/9	104.75	595.17	P2-8/12	80.79	459.03
C1/14	73.06	415.11	C3/2	25.07	142.44
C1/15	67.89	385.74	C3/5	76.53	434.83
C2			C3/7	82.34	467.84
P1-4/12	54.00	306.82	C3/8	58.46	332.16
P2-12/8	69.43	394.49	C3/11	42.60	242.05
C2/3	84.36	479.32	C3/14	108.78	618.07

These results are in accordance with those of Mansour et al. [23]. They used the promising mutant No.15 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.

In the construction of novel strains, protoplast fusion allows the combination of characteristics present in the parents. Although the isolation of a strain displaying specific traits may be a matter of good fortune. The advent of more specific means of manipulating genes has meant that protoplast fusion is no longer the principal method for strain improvement or construction. However, it allows the creation of strains embodying a range of characteristics from the parents or displaying polygenic traits that would be difficult to construct using more refined methods. Although the concentration of γ-decalactone obtained in the present study was lower than one reported production by Pagot et al. [24]. They were able to obtain γ-decalactone in high yields from ricinoleic acid methyl ester. At the end of the growth phase they transferred the concentrated biomass into an uracil limited medium where the biotransformation took place, after 75 hrs the culture broth yields 9.5 g/L of the product. This variation in γ-decalactone yield compared to the present study, the yields of γ-decalactone in all protoplast fusions (< 1 g/L) (Table 5), may be due to the incubation time. However, our results in accordance with Cardillo et al. [25] they used *Aspergillus niger*, *Pichia etchellsii* and *Cladosporium suaveolens* to produce γ-decalactone from castor oil. Common to all these processes is that the yields of γ-decalactone in all the fermentations with these yeasts were always very low (< 1 g/L).

Conclusion

It can be concluded that protoplast fusion between some superior *Y. lipolytica* mutants proved to be an effective technique to enhance γ-decalactone production. In a next step, fed-batch and continuous processes will be investigated in our laboratory for γ-decalactone production by selected *Y. lipolytica* fusants.

References

1. Endrizzi A, Pagot Y, Le CA, Nicaud JN, Belin JM. Production of lactones and peroxisomal β-oxidation in yeasts. Crit Rev Biotechnol **1996,** 16:301-329.
2. Gatfield IL. Biotechnological production of natural flavor materials. In: Flavor Chemistry: 30 Years of Progress ed. Teranishi R, Wick E, Hornstein I. **1999,** pp. 211-227. New York: Kluwer Academic, Plenum Publishers.
3. Schrader J, Etschmann M, Sell D, Hilmer J, Rabenhorst J. Applied biocatalysis for the synthesis of natural flavour compounds – current industrial processes and future prospects. Biotechnol Lett **2004,** 26: 463-472.
4. Siek TJ, Albin IA, Sather LA, Lindsay RC. Comparison of flavor thresholds of aliphatic lactones with those of fatty acids, esters, aldehyde, alcohols and ketones. J Dairy Sci **1971,** 54:1-9.
5. Mosandl A, Gunther C. Structure and properties of γ-lactone enantiomers. J Agric Food Chem **1989,** 37:413-418.

6. Lee SL, Cheng HY, Chen WC, Chou CC. Production of γ-decalactone from ricinoleic acid by immobilized cells of Sporidiobolus salmonicolor. Process Biochem **1998,** 33:453-459.
7. Wache Y, Aguedo M, Nicaud J-M, Belin J-M. Catabolism of hydroxyacids and production of lactones by the yeast *Yarrowia lipolytica*. Appl Microbiol Biotechnol **2003,** 61: 393-404.
8. Garcia E, Nicaud J-M, Belin J-M, Wache Y. Effect of acyl-CoA oxidase activity on the accumulation of γ-decalactone by the yeast *Yarrowia lipolytica*: A factorial approach. Biotechnol J **2007,** 2: 1280-1285.
9. Wache Y, Aguedo M, Choquet A, Gatfield I. Role of β-oxidation enzymes in the production of γ-decalactones from methyl ricinoleate. Appl Environ Microbiol **2001,** 67:5700-5704.
10. Fickers P, Fudalej F, Nicaud JM, Destain J, Thonart P. Selection of new over-producing derivatives for the improvement of extracellular lipase production by the non-conventional yeast *Yarrowia lipolytica*. J Biotechnol **2005,** 115: 379-386.
11. Blin-Perrin C, Molle D, Dufosse L, Le-Quere J-L. Metabolism of ricinoleic acid into γ-decalactone: β-oxidation and long chain acyl intermediates of ricinoleic acid in the genus *Sporidiobolus* sp. FEMS Microbiol Lett **2000,** 188: 69–74.
12. Dujon B, Sherman D, Fischer G, Durrens P. Genome evolution in yeasts. Nature **2004,** 430: 35-44.
13. Aguedo M, Beney L, Wache Y, Belin JM. Mechanisms underlying the toxicity of lactone aroma compounds towards the producing yeast cells. J Appl Microbiol **2003,** 94: 258-265.
14. Khattab A, Ibrahim G, Fatma N, Talkhan Osman F, Magda A. Abd El-Mageed El-Ghorab A. Genetic improvement of the β-oxidation pathway in the yeast *Yarrowia lipolytica* to increase the production of γ-decalactone. Austr J Basic Appl Sci **2012,** 6: 206-214.
15. Amaral PF, de-Almeida AP, Peixoto T, Rocha-Leao MH, Coutinho JA, Coelho MA. Beneficial effects of enhanced aeration using perfluorodecalin in *Yarrowia lipolytica* cultures for lipase production. World J Microbiol Biotechnol **2007,** 23: 339-344.
16. Adelaide B, Gomes N, Belo I. Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J Am Oil Chem Soc **2012,** 89: 1041-1047.
17. Spencer TF, Laud P, Spencer D. The use of mitochondria mutants in the isolation of hybrid involving industrial yeast strain. II: Use in isolation of hybrid obtained by protoplast fusion. Mol GenGenet **1980,** 179:651-654.
18. Farahanak F, Seki T, Ryu D, Ogrydziak D. Construction of lactose-assimilating and high ethanol-producing yeasts by protoplast fusion. Appl Environ Microbiol **1986,** 51: 362- 367.
19. Groguenin A. Wache Y. Escamilla, E. Aguedo M. Genetic engineering of the beta-oxidation pathway in the yeast *Yarrowia lipolytica* to increase the production of aroma compounds. J Mol Catal B **2004,** 28: 75-79.
20. Peberdy JF, Ferenczy L. Fungal protoplasts: Applications in biochemistry and genetics. **1985,** Marcel Dekker, Inc.New York.Basel.
21. Lin C, Hsieh P, Mau J, Teng D. Construction of an inter-generic fusant from *Schizosaccharomyces pombe* and *Lentnula edodes* for xylan degradation and polyol production. Enzyme Micro Technol **2005,** 36: 107-117.

22. Khattab A. Bazaraa. W. Screening, mutagenesis and protoplast fusion of *Aspergillus niger* for the enhancement of extracellular glucose oxidase production. J Ind Microbiol Biotechnol **2005,** 32: 289-294.
23. Mansour A.F. Fatma N. Talkhan El-Ghorab A. Olive oil as a precursor for γ-dodecalactone by biotransformation using *Yarrowia lipolytica* isolates. Bull Fac Agric, Cairo Univ, **2002,** 53: 427-442.
24. Pagot Y, Endrizzi A, Nicaud JM, Belin JM. Utilization of an auxotrophic strain of the yeast *Yarrowia lipolytica* to improve γ-decalactone production yields. Lett Appl Microbiol **1997,** 25:113-116.
25. Cardillo R, Fuganti C, Sacerdote G, Barbeni M, Cabella P, Squarcia F. Procede de production microbiologique de decanolide gamma (R) et d'octanolide gamma (R). European Patent No. **1990,** 0356291.