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OLIVE OIL AS A PRECURSOR FOR γ-DODECALACTONE BY BIOTRANSFORMATION USING *Yarrowia lipolytica* ISOLATES

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ABSTRACT

The main advantages of the *Yarrowia Lipolytica* (NRRL Y-17536) as well as some UV – induced mutants from it were evaluated for their ability to hydrolyze olive oil and β-oxidize the hydrolyzate in the medium in absence of co – oxidants.

The promising mutant No. 15, which overyielded its original strain in hydrolization of the oil, was used in protoplast fusion with the parental strain aiming to isolate high enzyme productive fusants. The culturing process takes place at a pH 6-7 and a temperature of 37°C for 7 days and eventually gives 4-hydroxydodecanoic acid. Cyclization or lactonization of the later by polyphosphoric acid at pH 1.5 and at 90-100°C results in the formation of γ-dodecalactone, which was identified by using GC / MS.

***Key words**: aroma biotechnology, biotransformation, lactones, olive oil , Yarrowia lipolytica.*

1. INTRODUCTION

Due to the growing market share of flavours in the world trade and the strong demand for natural products, including natural flavours, it was necessary to develop a number of biotechnological

solutions to compete with traditional synthetic chemistry for the production of flavours. In addition to the label "natural" characterizing the flavours produced via microbiological methods, biotechnological approach implies additional advantages, *e.g.*, chirality control on the end– product which affects its sensory properties, ability for scaled–up production, clean and cheap technology, and the suitability for the natural resources in developing countries (Krings and Berger, 1998). There are three possible approaches to obtain natural flavour chemicals via biotechnological methods or the use of so – called biocatalysis: plant cells and tissue mutants, fermentation and biotransformation. The latter approach can be performed either with whole cells or crude / isolated / purified enzyme systems with the addition of a main substrate which is converted into the desired product via enzymatic – catalyzed steps (Whitehead, 1998). The main advantage of the biotransformation technique is the using of inexpensive, readily available and renewable natural precursors, *e.g.*, fatty acids, which can be converted into more highly valued flavours, *e.g.*, lactones. *Yarrowia lipolytica* is a rather common fungus isolated from substrates containing lipids or proteins, such as dairy products, and is able to produce extracellular lipase. It has potential for utilization and conversion of industrial wastes containing lipids to food or flavours through providing fatty acids to be converted by microbial enzymes. Production of lipase from *Yarrowia lipolytica* is enhanced significantly by olive oil as a carbon – source (Gorzo and Revah, 1999; Felice *et al.*, 1997; Novotny *et al.*, 1988). The maximum activity was reached at pH 6-7 and 37°C (Destain *et al.*, 1997; Demnerova, 1994). Hadeball (1991) found that lipases play an increasing role in the conversion of hydrophobic compounds, especially lipids and within the fungus genus very different lipases are produced concerning their substrate specificity.

Lactones *e.g.*, γ-decalactone and γ-dodecalactone contribute to the flavour of many fruits such as strawberries, peaches and apricots, and also in milk products as well as some fermented foods (Gatfield, 1996). Microorganisms form these lactones by two possible pathways: de novo synthesis and the hydroxylation of fatty acids following β-oxidation (Gatfield, 1985; Hagedorn and Kaphammer, 1994; Imai *et al.*, 1991 and Janssens *et al.*, 1992). With respect to the latter, unsaturated fatty acids such as palmitoleic and oleic acidc were

expected to form γ-decalactone and γ-dodecalactone respectively, through hydroxylation followed by β-oxidation of the hydroxy fatty acid (Wanikawa *et al.*, 2000). Industrially, the use of unsaturated fatty acids, hydroxy fatty acids especially ricinoleic acid as well as castor oil to produce γ-decalactone is well known. However, nothing was reported concerning the presence of suitable and cheap substrate *e.g.*, essential oil, for the production of γ-dodecalactone. The main objective of this study was to convert unsaturated fatty acids of the olive oil to lactone precursors followed by lactonization using a suitable mineral acid, in order to obtain γ-dodecalactone *via* a commercial source. Bioconversion in this study depends on the mutation breeding or protoplast fusion technique to induce high productive isolates of lipase, which are obtained through different genotypes of *Yarrowia lipolytica*.

2. MATERIALS AND METHODS

2.1. Organism: *Yarrowia lipolytica* NRRL 17536, kindly provided by Northern Regional Research Lab., Department of Agrimutant, Illinois, USA, was used in this investigation.

2.2. Media and Solution

2.2.1. Minimal medium (MM): was used for mutant isolation and it has the following composition: yeast extract 3 g., malt extract 3 g., peptone 5 g., glucose 10 g. and 1000 ml distilled water. (Ogrydziak *et al.*, 1978).

2.2.2. Complete medium (CM): was used for mutant isolation and it contains: yeast nitrogen base without amino acids (Difco) as 0.67% and glucose as 1% at pH 4.5. (Ogrydziak *et al.*, 1978).

2.2.3. Fermentation medium for lipase production (FM): The medium contains the following composition: glucose 20 g., urea 2 g, olive oil 10 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 G., $CaCl_2$ 0.1 g, NaCl 0.1 g, H_3BO_3 0.5 mg, KI 0.1 mg, $FeCl_3$ 0.2 mg, $CuSO_4$ 0.04 mg, $ZnSO_4$ 0.4 mg, $MnSO_4$ 0.4 mg, myoinositol 4 μg, biotin 8μg, thiamin 200 μg and 1000 ml. of distilled water at pH 4.0. (Gorzo and Revah, 1999).

2.2.4. Regeneration medium: It consists of: yeast extract 0.5 g, peptone 0.5g, glucose 10g, KH_2PO_4 0.75 g, $(NH_4)_2SO_4$ 0.75 g, $MgSO_4 . 7H_2O$ 0.5 g, sorbitol 5 g and 500 ml. distilled water (Takagi *et al.*, 1983).

2.2.5. Protoplast fusion buffers: pretreatment solution (Spencer *et al.*, 1980);protoplasting buffer(Spencer *et al.*,1980); polyethylene glycol solution(Farahanak *et al.*,1986) and potassium phosphate buffer(10 mM K_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl) at pH 7.0.

2.3. UV – light mutagenicity: A Philips T– UV–30 W lamp (type no. 57413P140) was used as a source of UV– irradiation in dark at a distance of 20 cm. Cells of the organism were irradiated in open –dishes with agitation for 0, 5, 10 and 15 minutes. For avoiding photoreactivation, treated cells were kept in dark for about 2 hours after which appropriate dilutions were inoculated on CM plates. The growing colonies were counted against the control plates of the same dilution. Colonies, which gave growth on CM but not on MM plates, were considered auxotrophs. In addition, some morphological mutants were isolated to be tested for lactone formation.

2.4. Fermentation and Bioconversion

The mutants were carried out in 50 ml of FM in 250 ml Erlenmyer flasks for 7 days with agitation at 200 rpm, 35 °C and pH 7.0. Concentrations of Tween–80 between 0.5 and 2 g/l were added to activate the extracellular lipase production. Olive oil, oleic acid or linolenic acid were individually added as substrates at a rate of 10 g/l in the fermentation media.

2.5. Protoplast fusion and isolation of fungus fusants

. Protoplast formation was carried out according to the method described by Spencer *et al.*, (1980). The induced mutant no. 15, which proved to be the highest lactone precursor formation as found in this study, was chosen to be used in protoplast fusion with the original strain. These two isolates were subjected to different antibiotic stress to determine their resistance or sensitivity to each antibiotic. Streptomycin showed inhibition effect against the mutant strain(no.15)

but not on the W.T., while oxytetracycline inhibited the growth of the W.T. but did not affect mutant no.15. Protoplasts from each of the W.T. (no.25) and the mutant no.15 were mixed and centrifuged at 3000 rpm for 5 min and the supernatant was removed. Polyethylene glycol (2 ml) was added and mixed gently with protoplasts until they were resuspended. The mixture of the protoplasts was incubated up to 40 – 60 min at 30°C, and then diluted with 0.65 M KCl to 10^{-1}. Samples of 0.2 ml were plated on 20 ml of regeneration medium and incubated at 30°C for 2 days. The obtained regenerated colonies, which proved to be resistant to both antibiotics, were considered fusants. Five stable fusants were selected and tested on fermentation media containing olive oil for their lactone precursor formation.

2.6. Isolation and Lactonization

At the end of the fermentation period, cells were removed by centrifugation and the pH of the medium was adjusted to 1.5 by the addition of mineral acid (polyphosphoric acid, Merck Darmstadt, Germany) and the mixture was heated to 100°C for 5-10 min in order to complete the lactonization. Extraction of the γ - dodecalactone formed was carried out using 20 ml of diethyl ether (Merck, Darmstadt, Germany). The organic layer was separated, dried over anhydrous sodium sulfate and concentrated to about 0.5 ml using a Vigreux column (50 cm x 1 cm) and the sample was examined by GC/ MS analysis.

2.7. Sensory Evaluation

The sensory analysis was carried out under the conditions specified by the international standards (International Standardization Organization, ISO); general guidelines after ISO 6658-1985; in a test room provided with standard test booths (ISO 8589-1988); the assessors were selected, trained and monitored after the general standard (ISO 8589-1988); unstructured graphical scales (ISO 4121-1988) were presented as straight lines 100 mm long, provided with descriptors on either end (odour intensity: 0 mm = very weak, 100 mm = very strong; odour acceptability: 0 mm = very little agreeable, 100 mm = very agreeable). The sensory profile was based on free choice profiling, and the following descriptors were retained (out of 32 collected descriptors) : 1 = roasted, bread crust, roasted peanuts,

ginger bread; 2 = sweet, caramel, bitter; 3= pasty, floury, bread crumb; 4 = fruity; 5 = sharp, pungent, burning; 6 = fatty, oily, buttery; 7= earthy, musty, moldy; 8 = others – specify which); in the profile evaluation: 0 mm = absent, 100 mm = very strong. Odour profiles were tested by sniffing from ground wide-neck glass bottles.

2.8. Gas Chromatography– Mass Spectrometry Aanalysis (GC/ MS)

GC – MS analysis of the cyclized or lactonized products after extraction, was performed on a Varian gas chromatograph interfaced to Finnigan SSQ 7000 mass selective detector (MSD) with ICIS V2.0 data system for MS identification of the GC components. The column used was DB – 5 (J & W Scientific, Folsom, CA) cross – linked fused silica capillary column (30 m, 0.25 mm i.d.) coated with polydimethylsiloxane (0.5µm film thickness). The oven temperature was programmed from 50°C for 3 min isothermal, then heating by 7°C / min to 250°C, and isothermally for 10 min at 250°C. Injector temperature was 200°C and the volume injected was 1µL. Transition – line and ion source temperatures were 250°C and 150°C, respectively. The mass spectrometer had a delay time of 3 min to avoid the solvent peak and then scanned from m/z 40 to m/z 350. Ionization energy was set at 70eV. Identifications were based on the comparison with the MS computer library (NIST – Software package, Finnigan) and on the respective retention indices. The separated components were identified by matching them with the National Institute of Standards and Technology (NIST) mass spectral library data, comparison of the Kovat's indices with those of authentic components and with published data (Adams, 1995).

3. RESULTS AND DISCUSSION

Upon treatment of *Yarrowia lipolytica* NRRL 17536 with UV light at different exposure times, 24 different stable mutants were chosen, 5 of which from the treatment for 5 min., 3 from the 10 min and 16 from the 15 min treatments. Figure (1) shows some colonies of these mutants.

The activity of different mutants (24) along with their W.T. strains were tested individually for the formation of γ-dodecalactone

precursor using olive oil, oleic acid and linolenic acid as substrates. After 168 hours of fermentation, the mutant broth was taken and subjected to *in–situ* lactonization followed by sensory evaluation. The promising products were analyzed *via* GC and GC / MS.

3.1. Sensory Properties

Table (1) demonstrates the sensory profile of the concentrates formed after lactonization of different mutant broth. A trained panel using a scaling procedure quantified the whole flavor as well as the intensity of eight part qualities (sensory notes). Three-fold repetition and use of standard gave reproducibility of the tests. The mutant type as well as the substrate used, as shown in Table (1), significantly affected the odor acceptability, *e.g.*, mutant no. 15 using olive oil as a substrate showed the highest record, however the same mutant using oleic or linolenic acids as substrates gave a lower acceptable records. Gorzo and Revah (1999) reported the inhibitory effect of oleic acid on the lipolytic action of *Yarrowia lipolytica* fungus, but surprisingly we detected the same effect using linolenic acid as a substrate for this organism. The same phenomenon was observed for the concentrates prepared *via* mutant no. 9. Among the numerous concentrates obtained and examined, those of mutant no. 16 using oleic acid and linolenic acid were recorded the lowest acceptable records.

The odor intensity was higher in most concentrates, whereas lower intensities were observed in the concentrates obtained through mutants no. 7, 9 and 23 using linolenic acid as a substrate; this may be due to the lower activity of such mutants towards the formation of aroma precursors.

More pronounced differences were observed in the odor profile. Intensity of the sweet – fruit flavor notes recorded the highest grades in the concentrate obtained *via* mutant no. 15 using olive oil as a substrate, apparently, due to the formation of γ-dodecalactone. In general, fruity, fatty and sweet notes were found stronger in concentrates prepared *via* mutants broth, which depend on the olive oil as a substrate. However, the same notes were weaker in those obtained through mutants depending on oleic and linolenic acid as substrates. This seems to be reasonable, since both oleic acid as well as linolenic acid inhibited the organism activity, and only esters were formed due to acidification during the lactonization step. Other

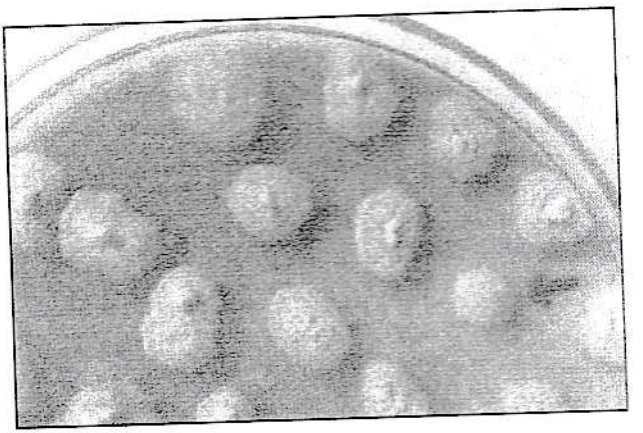


Figure (1) Some indced mutants after UV treatment with different growth and shape colonies (Arrow refers to muntant No. 15).

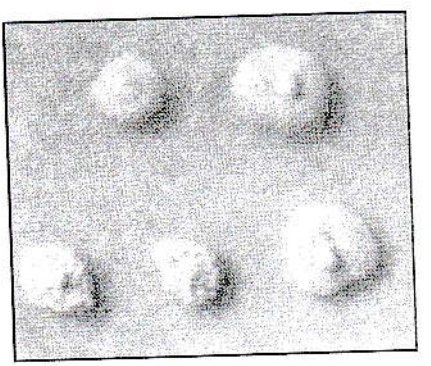


Figure (2) : Colnies of five chosen fusants

descriptors gave insignificant results, as the ratings were too low; therefore they are not included in Table (1).

Upon protoplast fusion technique (Figure 3) between the wild type strain (no. 25) and the promising mutant (no. 15), 42 different fusants were obtained and tested for the formation of γ-dodecalactone precursor in the supernatant of the fermentation medium. The most productive five fusants were chosen (Figure 2). Table(2) demonstrates the sensory profile of the concentrates formed after lactonization of these five mutants broth using olive oil as a substrate.

Table (1): Sensory evaluation of the concentrate samples of 5 selected mutants along with the W.T. (No. 25) using olive oil as a substrate (Results expressed in p.c. of the graphical scale)

Characteristic	No. 15	No. 25	No. 9	No. 23	No. 7	No. 16
Odour intensity	88.6	72.3	74.5	65.4	70.8	69.0
Odour acceptability	93.1	85.7	90.1	82.4	88.2	89.0
Odour profile:						
Roasted, bread crust	1.1	2.4	1.7	1.9	2.2	1.4
Sweet, caramel	80.4	29.5	37.3	27.1	20.5	17.9
Fruity	90.2	66.1	60.8	62.4	59.6	68.4
Pasty, bread crumb	1.0	1.2	1.1	1.0	1.7	1.2
Fatty, buttery	26.6	48.8	38.5	45.4	44.8	40.7
Earthy, mouldy	1.2	3.9	2.5	3.3	2.4	1.9

3.2. Composition of volatiles and their precursors

As mentioned above, only the promising concentrates, according to the sensory evaluation, were subjected to GC and GC/MS analyses. The concentrate formed through mutant no. 15 using olive oil, as a substrate was the most promising one. According to the GC and GC / MS analyses, two main components were identified in this concentrate; the first showed the following apparent fragments:

m/z 273 = M, 256 (palmitic acid – OH group), and m/z 213 which represents the hydrocarbon moiety (Figure 4a). From these results, it was evident that the product formed by mutant no. 15 using olive oil as a substrate was 10-hydroxypalmitic acid. In Figure (4b) mass spectrum of the second component detected in this concentrate with

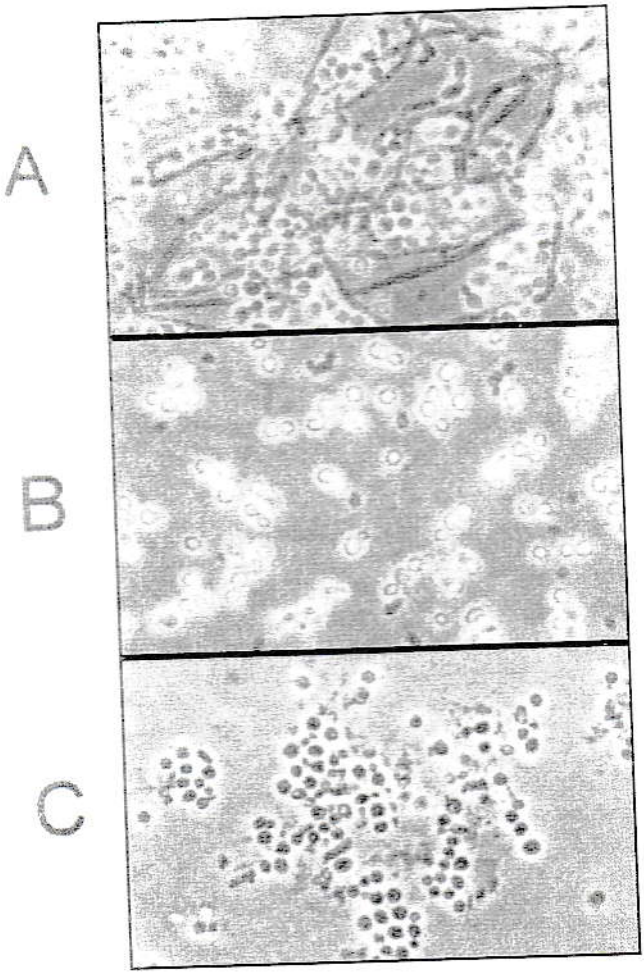
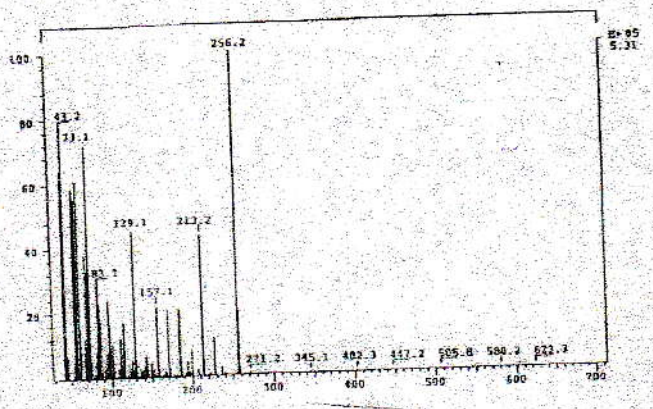
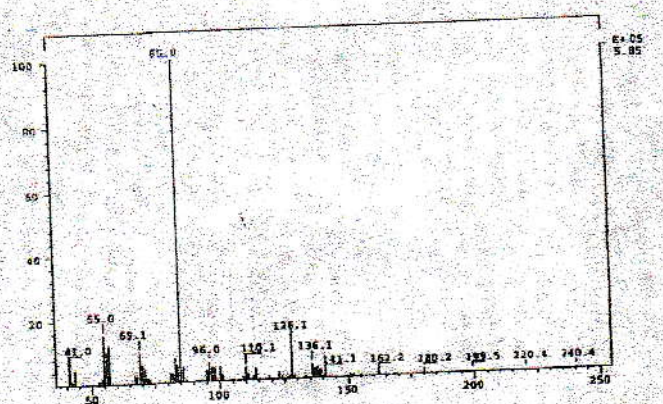


Figure (3): A: The *Yarrowia lipolytica* before snail lytic enzyme.
B: The cells after enzyme.
C: Fusant cells.



(a)



(b)

Figure (4) : Mass spectrum of a) 10-hydroxypalmitic acid . b) Y-dodecalactone.

GC purity of 94% is presented. It is characteristic with a base peak at m/z 85, which represents the tetrahydropyranoyl cation. The above results are in agreement with those obtained through the control mutant no. 25. Quantitation study for the γ-dodecalactone formation indicated that the level of this study compound was progressively increasing with increasing the fermentation period. A concentration of 788 mg/l of γ-dodecalactone was obtained from the fermentation broth of mutant no. 15.

Table (2): Sensory evaluation of the concentrate samples of 5 different fusants using olive oil as a substrate (Results expressed in p.c. of the graphical scale).

Characteristic	No. 34	No. 38	No. 35	No. 27	No. 29
Odour intensity	56.8	31.2	18.9	61.4	70.4
Odour acceptability	66.4	42.5	31.8	55.7	79.3
Odour profile:					
Roasted, bread crust	0.0	0.6	1.2	2.1	1.8
Sweet, caramel	33.1	18.7	8.6	31.4	23.7
Fruity	61.4	31.1	23.8	70.1	81.6
Pasty, bread crumb	1.0	0.0	1.6	1.1	2.1
Fatty, buttery	41.2	38.1	40.6	31.2	43.0
Earthy, mouldy	0.0	2.0	1.5	2.4	3.0

There are two possible pathways to the biosynthesis of the precursor γ-dodecalactone. One results from the fact that oleic acid seems to be a genuine precursor of the γ-lactones. In this respect, Haffner and Tressl (1996) suggested a new biosynthesis pathway depending actually on the enantioselective R-12-hydroxylation of oleic acid as an initial step followed by β-oxidation leading to the lactone. The other pathway is through the lactonization of 10-hydroxypalmitic acid, which was found to be produced from palmitoleic acid (Wanikawa *et al.*, 2000).

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التحويل الحيوي لزيت الزيتون الي مواد وسطية في صناعة اللاكتونات كمكسبات طعم و رائحة بواسطة فطر *Yarrowia lipolytica*

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قسم كيمياء مكسبات الطعم والرائحة – * قسم الوراثة والسيتولوجي – المركز القومي للبحوث – الدقي – الجيزة

ملخص

تعتبر عملية التحويل الحيوي من انسب الطرق صناعيا لانتاج مكسبات الطعم و الرائحة من حيث اعتمادها علي التحويل الانزيمي لبعض المواد الصناعية و المتوفرة اقتصاديا بشكل واسع مثل الاحماض الدهنية والزيوت وذلك بتحويلها الي مواد وسطية يمكن استغلالها في صناعة مركبات مكسبة للطعم و الرائحة ذات قيمة عالية مثل اللاكتونات وهي المميزة كرائحة للفواكه.

تم في هذا البحث الحصول علي حمض 4-hydroxydodecanoic وهو مادة وسطية في صناعة اللاكتونات وذلك بواسطة فطر *Yarrowia lipolytica* و الذي يقوم بتكسير و اكسدة زيت الزيتون للوصول الي هذا الحمض. وقد امكن الحصول علي نسبة عالية من الحمض بعد سبعة ايام عند درجة حرارة ٣٧ م و اس هيدروجيني ما بين ٦– ٧ .

وجد ان السلالة رقم ١٥ و الناتجة من التهجين الوراثي للسلالة الاصلية قد اعطت اعلي انتاجية انزيمية و علي هذا الاساس فقد تم تهجينها من اجل الحصول علي سلالات اخري ودراسة اثر ذلك التهجين علي الانتاجية الانزيمية.

تم الحصول علي اللاكتونات المكسبة للطعم و الرائحة بواسطة حولقة حمض 4-hydroxydodecanoic عند درجة اس هيدروجيني ١،٥ و درجة حرارة ٩٠–١٠٠ م و باستخدام حمض عديد الفوسفوريك. هذا و قد تم التعرف علي درجة نقاء و تركيب تلك اللاكتونات تحليليا بواسطة جهاز الكروماتوجرافيا الغازية– طيف الكتلة.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (٥٣) العدد الثالث (يوليو ٢٠٠٢): ٤٢٧–٤٤٢.