

Esterase profiling and molecular identification of yeasts isolated from different environmental samples from Morocco

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Abstract

One hundred and six fungal strains were isolated from different environmental samples (fresh olive oil cake, exhausted olive oil cake, black olive, rancid butter samples, rotten bread and Roquefort) collected from the region of Meknes, Morocco (coordinates: 33°53'42"N 5°33'17"W). Yeast isolates were tested for their esterase production ability using a qualitative method based on Tween agar plate assay. Enzymatic activity was also confirmed by a quantitative method relying on esterase production in liquid medium (6 days at 28°C with shaking).

Molecular characterization of the selected esterase-producing yeasts was performed by sequencing the internal transcribed spacer 1 (ITS1), 5.8S and ITS2 region of the rDNA.

A total of five different species were identified in this study: *Candida aaseri* (LE.26, LE.27 and LE.31 strains), *Wickerhamomyces anomalus* (LE.106, LE.112 and LE.115 strains), *Metschnikowia rancensis* (LE.153 strain), *Pichia* sp., (LE.102) and *Rhodotorula mucilaginosa* (LE.171 strain). Esterase production in *C. aaseri* and *W. anomalus* was found to be strain-dependent, while for *M. rancensis* this represents the first study reporting this species as an esterase producer.

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Contributions: MH conceived the study and supervised the experimental work regarding collection, isolation and phenotypic identification of fungal isolates; LEA, MH and OR designed the molecular study; OR and GC supervised the work relating molecular identification of selected fungi; LEA and CB performed lipase production experiments and determined enzymatic activity; LEA and FS carried out molecular analysis and interpreted the data with OR; LEA wrote the main manuscript text and prepared figures and tables. MH, CG and OR participated in discussions and provided suggestions. All the authors reviewed and approved the final manuscript.

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Introduction

Esterases (E.C.3.1.1.1) are enzymes that generally hydrolyse ester bonds¹ in short-chain fatty acids,² showing their highest activity toward the soluble state of its substrate.³ Esterases are a group of enzymes with apparent potential as biocatalysts used by pharmaceutical and chemical industries⁴ as well in agro-food and dairy industries where esterases contribute to the flavour development of some foods, in particular cheeses and wines.⁵

Esterases are produced by various microorganisms including bacteria⁵⁻⁹ and fungi^{10,11} and are very attractive because the costs associated with their production are reduced by the fact that microbes are easy to grow, maintain and manipulate. Therefore, there is a growing interest in discovering new microbial esterases with novel or improved physicochemical properties for industrial applications.

Traditionally, fungal identification has mainly been based on physiological and morphological proprieties¹² but today, molecular characterization is necessary and it is currently used for taxonomic recognition of the isolated species. In general, molecular identification of fungi is based on sequence analysis of specific genetic markers such as the partial sequencing of the large subunit (LSU) of the rRNA gene (D1–D2 domains), γ -actin (ACT), translation elongation factor 1- α (TEF1 α), partial β -tubulin II (TUB2) including the whole internal transcribed spacer regions (ITS1-5.8S-ITS2).¹³ Of note, this latter marker is considered as the universal DNA barcode for the fungal kingdom and therefore ITS-sequencing is often used as primary tool for species delineation.¹⁴

The aim of this study was to screen for new, potential esterase-producing fungi by examining different environmental samples collected in the region of Meknes (Morocco) and to identify the recovered fungal species following the analysis of the ITS1-5.8S-ITS2 region according to previous studies.¹³

Materials and Methods

Sampling and isolation

Different environmental samples (fresh olive oil cake, exhausted olive oil cake, black olive, rancid butter (n° 3 samples), rotten bread and Roquefort cheese) were collected in sterile containers and analyzed for the detection of esterase-producing fungi.

Except for rancid butter, 20 grams of each sample were suspended in 20 mL of sterile saline solution and homogenized using a vortex for 5 min. Subsequently, ten-fold serial dilutions were prepared and aliquots of 100 μ L of each dilution were plated in potato dextrose agar (PDA) and incubated at 30°C for three days. After the incubation time, yeast colonies were purified at least twice and maintained in PDA.

For the three rancid butter samples, 10 grams of each sample were added to 10 mL of sterile saline solution and incubated in water bath at 45°C for 30 min. Then, the samples were agitated for 30 sec by vortexing and only aqueous phase was used for microbiological analysis.

Screening of yeasts for extracellular esterase production on solid agar

Qualitative screening of extracellular esterase producing yeasts was performed using the Tween agar (peptone, 10 g/L; NaCl, 5g/L; CaCl₂, 0.1g/L; agar, 15 g/L; Tween 20, 10 mL/L; and distilled water, 1000 mL) plate assay.¹⁵ The agar plates were spot inoculated by the isolated yeasts on the surface and then incubated at 28°C for four days. Esterase production was detected by the appearance of a clear halo around the spot inoculum.

The ratio between the halo (H) diameter and cell colony (C) diameter (H/C index) was determined and used as a measure of esterase production. A H/C value of 1 indicated no esterase production while higher H/C values were considered as indicative of esterase secretion.¹⁶

Screening of the selected isolates for lipase production using submerged fermentation

The esterase producing yeasts on solid agar were further screened for esterase production in submerged fermentation medium. Selected yeasts were grown in 2% malt extract for 24h at 28°C and then inoculated in the fermentation medium (10 g/L yeast extract; 2 g/L KH₂PO₄; 0.3 g/L MgSO₄·7H₂O; 12 g/L Na₂HPO₄; 0.3 g/L CaCl₂·H₂O; 2% Tween 20; final pH adjusted to 6.0±0.2). The experiments were repeated three times.

Extracellular esterase production

The culture was grown in 100 cm³ Erlenmeyer flasks containing 50 cm³ of the production medium inoculated with 1% (v/v) (0.5 cm³) of cell suspension and incubated at 28 °C on a rotary shaker (120 rpm) for 6 days. Then the fungal cells were removed with centrifugation at 6000 rpm at 4 °C for 20 min and supernatants were filtered through 0.22 μ m pore-sized filters. The filtrates were tested for esterase activity.

Assay for esterase activity

For esterase assay, the substrate solution was prepared by adding 100 μ L of the p-nitrophenyl acetate (p-NPA) solution (3.6 mg p-NPA from Sigma Aldrich, Italy, dissolved in 2 mL of propane-2-ol and acetonitrile 1:1) to 75 μ L of water and 800 μ L of 0.05 mol/L Tris-HCl buffer (pH 8.0). The esterase assay was carried out at 25°C, by adding 25 μ L of the enzyme extract to the sub-

strate solution. The reaction was stopped after 20 min by incubating the mixture on ice. The absorbance was spectrophotometrically measured at 405 nm against an enzyme-free control.

One lipase International Unit (IU) was defined as the amount of enzyme that liberates 1 μ mol of p-nitrophenol per min, under the described assay conditions.

Molecular identification of the selected esterase-producing yeasts

The molecular identification of the selected esterase producing yeasts was done respecting the workflow in Figure 1. The DNA was extracted using the high-speed cell disruption method as described by Müller *et al.*¹⁷

The ITS1-5,8S-ITS2 region was amplified by polymerase chain reaction (PCR) using the DreamTaq™ PCR master mix (Fermentas, Milan, Italy) and the following universal primers: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCT-TATTGATGC).¹⁸

All PCRs were performed in a MyCycler thermal cycler (Bio-Rad, Milan, Italy) with initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s, with a final elongation step of 10 min at 72°C. Amplicons were separated and visualized by 1.5% agarose gel electrophoresis and then purified using the QIAquick PCR purification kit (Qiagen, Milan, Italy). Sequencing was performed at Eurofins Genomics (www.eurofinsgenomics.eu) using the ITS1 forward primer.

For taxonomic recognition of our isolates, the DNA sequences were compared with those available in the GenBank database using the BLAST (Basic Local Alignment Search Tool) algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Further phylogenetics analysis were conducted in MEGA7 software (www.megasoftware.net).

Results

Isolation and screening of esterase producing yeasts

In this study, a total of 106 fungal strains were isolated from the examined environmental samples. Nine of the yeasts showed a clear zone of precipitation around the inoculum spots consistent with the production of extracellular esterases. The H/C ratio for these nine isolates ranged from 1.77 to 3.57 whereas for the other isolated yeasts the H/C index value was = 1 (Table 1).

Lipase production using submerged fermentation

The following step of this study was intended to confirm results obtained with Tween agar plates assay. A submerged fermentation, including the determination of the esterase activity, was carried out using the 9 esterase-positive yeasts previously recovered (Figure 2).

The results obtained by examining the extracellular esterase activity from these strains are listed in Table 1. The maximum activity (243.64 UI \pm 1.02) was obtained by the LE.26 strain while the LE.31 strain showed the lowest (168.36 UI \pm 0.75).

Identification of extracellular esterase producing yeasts

The sequence comparison using the BLAST program and the GeneBank database revealed that LE.26, LE.27 and LE.31 strains had 100% similarity to *Candida aaseri* rDNA, while the LE.106, LE.112 and LE.115 strains showed 100% similarity to

Wickerhamomyces anomalus. The LE.153 strain had 98% similarity to *Metschnikowia rancensis* while the LE.102 strain was identified only at the genus level with a 99% similarity to *Pichia* sp. The remaining LE.171 strain was identified as *Rhodotorula mucilaginosa* (99% similarity).

The oligonucleotide sequences obtained in this study have

been submitted to Genbank under the following accession numbers: MH807456, MH807448, MH807488, MH807490, MH807459, MH807460, MH807457, MK131396 and MH807929. All the 9 strains have also been deposited in the Westerdijk Fungal Biodiversity Institute, CBS culture collection (www.westerdijkinstitute.nl), under the CBS accession numbers listed in Table 1.

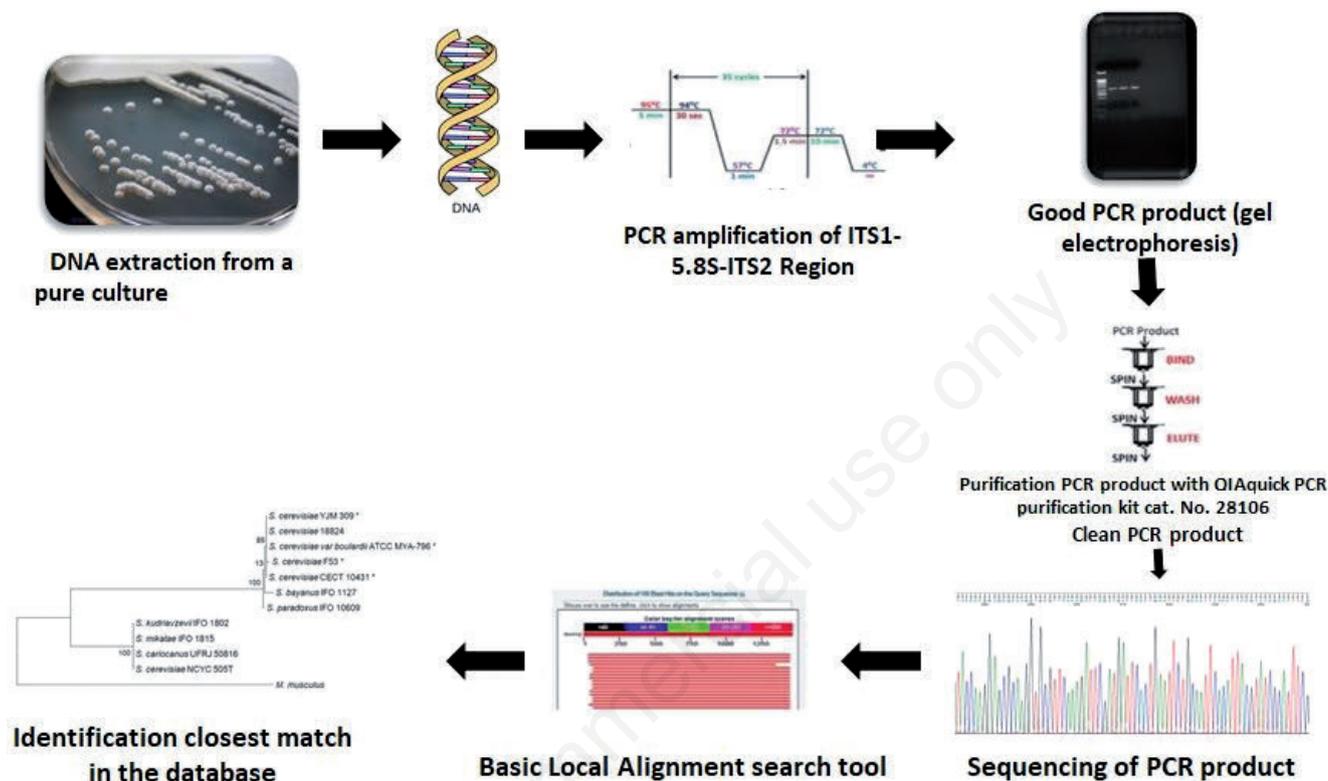


Figure 1. Molecular identification’s workflow of the selected esterase-producing yeasts using ITS-sequencing. PCR, polymerase chain reaction; ITS, internal transcribed spacer.

Table 1. Halo (H) diameter and cell colony (C) diameter (H/C index) of yeast isolates showing esterase production on solid Tween agar medium.

Isolates	H/C	Esterase activity
LE.26 (CBS 15940)	3.57	243.64 UI±1.02
LE.27 (CBS 15941)	3	190.5 UI±7.41
LE.153 (CBS 15946)	3	200.21 UI±1.96
LE.102 (CBS 15943)	2.66	193.28 UI±1.04
LE.106 (CBS 15944)	2.5	193.64 UI±1.86
LE.171 (CBS 15947)	2.5	196.21 UI±0.63
LE.115 (CBS 15997)	2.35	233.57 UI±2.16
LE.112 (CBS 15945)	2	200.14 UI±1.59
LE.31 (CBS 15942)	1.77	168.36 UI±0.75
Other isolated yeasts	1	-

CBS, Central Bureau of Fungal Cultures (the name has been changed to Westerdijk Institute, or Westerdijk Fungal Biodiversity Institute. Despite the change, the collection maintained by the institute remains the CBS collections and the use of CBS numbers for the strains is still valid).

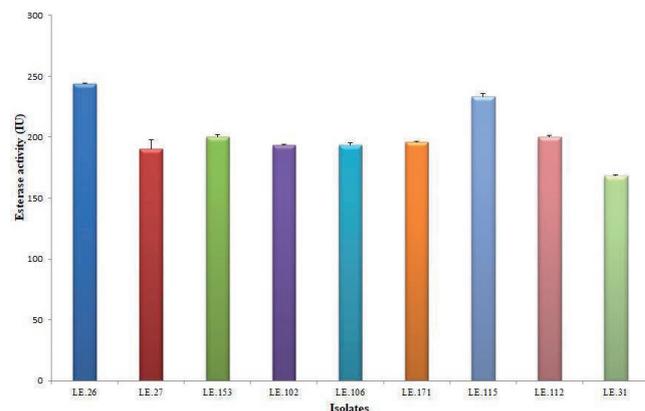


Figure 2. Esterase activity of selected fungal isolates using submerged fermentation performed for 6 days at 28°C with shaking. IU, lipase International Unit.

Discussion

In this study, among 106 isolated fungi, 9 esterase producing yeast strains were isolated from fresh olive oil cake, exhausted olive oil cake, black olive and rancid butter collected from the region of Meknes in Morocco. Many methods of esterase and lipase detection in agar plates exist in the scientific literature; the choice of the method depends on the requirements of the researcher. Fatty acid esters of polyoxyethylene sorbitan (Tweens) have been the most used substrates for the detection of microorganisms producers of esterase in agar media.^{19,20} The method is based on the precipitation of the fatty acids, released after hydrolysis of tween, that bind with the calcium incorporated into the medium, forming a calcium complex visible as crystals around the inoculation spot.²¹

The LE.26, LE.27 and LE.31 strains have been identified as *C. aaseri* and showed a variable esterase activity based on the obtained profiles. The strain LE.26 had the highest H/C ratio and highest esterase production in the liquid medium. *C. aaseri* has already been reported as a lipase producer,²² but since the esterase activity appears to be strain-dependent, it will be interesting to investigate other strains of this species in order to select strong esterase producers that could be used for the development of biotechnological processes.

The LE.106, LE.112 and LE.115 strains, identified as *W. anomalus*, also showed different esterase activity profiles. Like *C. aaseri*, also *W. anomalus* has been reported as lipase producer,²³ but, to our knowledge, it has never been tested for esterase production so far.

One interesting result obtained in this study was the isolation of a new esterase producer identified as *M. rancensis*. This species was the third highest esterase producer in this study and could represent a new source of esterase from microbial origin.

The LE.171 strain was identified as *R. mucilaginosa* and was found to produce esterase using both qualitative and quantitative methods. These results confirm previous published data showing lipolytic activity by strains belonging to the same species.²⁴

Conclusions

We showed that nine yeasts strains isolated from different environmental samples display esterase activity through both qualitative and quantitative analysis. One strain (*M. rancensis*) has been reported for the first time as a novel esterase producing yeast, highlighting that the fungal kingdom could represent an important source of biotechnologically important enzymes. However, further investigations are necessary to characterize the physicochemical properties of esterases secreted by our fungal strains recovered in this study.

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