Isolation and characterization of lipolytic yeasts derived from traditional tanneries of Fez Medina

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Abstract

Lipases are a group of enzymes which catalyse hydrolysis of long chain triglycerides into glycerol and fatty acid. They are positioned only after proteases and carbohydrases in world enzyme market and contribute to 5% of enzyme market. They are in plants, animals and microorganisms and are consequently classified as plant, animal and microbial lipases. Lipases produced by microorganisms are increasingly studied for the development of enzyme technology. This study is the first work showing lipase activity produced by yeasts isolated from Moroccan traditional tanneries. The kinetics of lipase activity of the three yeasts; *Trichosporon aashii, Trichosporon asteroids* and *Trichosporon coremiiforme* combined with the evolution of the biomass was studied. This allowed us to reveal two major phases of production of this enzyme.

Key words: yeast, lipase, *Trichosporon aashii, Trichosporon asteroids, Trichosporon coremiiforme*.

Introduction

Lipases (EC 3.1.1.3) have the ability to catalyze several reactions as well as hydrolysis, esterification and transesterification. For these reasons, they are the most widely used enzymes in organic synthesis (Elibol and Ozer, 2000). The Promising areas for application of lipases include biodegradation of plastics such as polyhydroxyalkanoates (PHA) and polycaprolactone (PCL) (Gombert *et al.*, 1999) and resolution of racemic mixture of optically active compounds (Reetz, 2002). Lipases are found in a wide range including animals, plants and microorganisms (Jaeger and Eggert, 2002). Among them, microbial enzymes are often more useful and more stable and their production is more convenient and safer.

Microbial enzymes are currently attracting an enormous attention because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995). Among all microorganisms yeast lipases are preferred enzymes in pharmaceuticals because of their enantiospecificity (Fariha *et al.,* 2006). At this moment, nonstop demand for greatly active lipase with appropriate properties encourages the research for the new enzyme sources.

Despite the particularity of the microbial enzymes, only about 2% of the world's microorganisms have been tested as enzyme sources. The extreme

environments as source of isolation and selection of useful microorganisms have been highlighted (Bull *et al.*, 1992) and the progress in this area has been possible with the isolation of large number of microorganisms from different ecological zones of the earth and subsequent extraction of useful enzymes from them (Antrankian *et al.*, 1987).

Our goal is to isolate the yeast of CHOUARA traditional tanneries of fez medina. The yeasts isolated were screened for lipase production and their lipolytic activity was studied.

Materials and methods

Sample collection

This study focused on CHOUARA tanneries localized in the medina of Fez. The samples have been taken from treatment ponds. These basins are grouped into four categories respectively specializing in the treatment with lime, pigeon droppings, the bran and the tanning basin. The samples are placed in bottles for physical-chemical assays. Those intended for microbiological analysis were collected from other sterile vials and transported to the laboratory promptly.

Physical-chemical characterization of samples

The physical-chemical characteristics of our sample were investigated. The temperature, pH, dissolved oxygen, chemical oxygen demand (COD), biological oxygen demand (BOD_5) and conductivity are determined. pH and temperature parameters are measured on site with a pH meter ORION type that indicates the pH and temperature of the different stages of tanning. The concentration of dissolved oxygen in different samples of water from tanneries was determined in situ by a portable oximeter. Biological oxygen demand and chemical oxygen demand (BOD5 and COD) are measured. BOD_5 is the amount of oxygen required by microorganisms to oxidize all organic material from a sample of water maintained at 20°C in the dark for 5 days. This parameter is expressed in milligrams of oxygen required for five days to degrade the organic matter contained in a liter of water. The measurements are performed in the laboratory by a BOD meter type Oxi-top (IS6). COD is the oxygen consumption required for complete oxidation of organic matter. It is expressed in grams of oxygen per liter of sample. The COD meter used for the oxidation of organic matter is type hot RECORD T5A FUSES. The determination of COD is carried out by powerful oxidant: potassium dichromate. The principle is based on oxidation to a boil (150°C for 2 hours) of reducing substances by an excess of potassium dichromate in sulfuric acid (H_2SO_4), and in the presence of silver sulfate as catalyst and sulphate of mercury as complexing of chloride. At the end of the reaction, the COD is measured by taking a suitably diluted sample before oxidation. The optical density of the sample is obtained by spectrophotometry at a wavelength of 585 nm. COD values are measured using a spectrophotometer type UV/ Visible. Finally electric conductivity was measured. The unit is milli-siemens per centimeter (mS/cm). The electrical conductivity of water samples from tanneries are measured by a conductivity meter Type WTW model LF318/set.

Isolation of yeast from CHOUARA tannery

Microorganism screened and studied in this work were isolated from CHOUARA tannery. The samples were diluted 10-fold and 0.1 ml was spread on pre-solidified YPG medium agar. The stock cultures were labeled, incubated at 30° C for 48 hours and stored at 4°C until use and a replicate was stored in sterile 20% glycerol solution at −80°C.

Screen of lipase producing yeast

Visualization of lipolytic activity on solid media determined by using dye Victoria blue B. Initial screening of lipolytic microorganisms from Tanneries of Fez was carried out using a plate assay in a medium containing triacylglycerol. The solid medium contains 5% olive oil, 1% nutrient broth, 1.5 g agar and 0,01% Victoria blue B. The culture plates were incubated at 30°C, lipolytic activity is observed directly by changes in the appearance of the substrate, which are emulsified mechanically in various growth media and poured into a Petri dish. Lipase production is indicated by the formation of clear blue halos around the colonies grown on triacylglycerol containing agar plates.

Molecular characterization and phylogenetic analysis of yeast strains

Molecular characterizations have been used. Microbial material was scraped from pure cultures and DNA extracted using thermal shock. Yeast identification was based on the ribosomal RNA 5.8 S using specific primers of the preserved areas. The ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers allow the amplification of 5.8 S rRNA region located between the 18 S and 28 S rRNA (Redecker, 2000). Genomic DNA was extracted from bacterial strains using thermal shock (Sambrook *et al.*, 1989). The PCR mixture contained 1.5 mM MgCl2, 200 µM of each dNTP's (Promega, Madison, USA), 1 µM of each primer (Metabion, Bangalore, India), 4 µl of Taq buffer (5X) and 1 unit of Taq polymerase (GoTaq Gold, Promega, Madison, USA). To this mixture, 2 µl of the DNA template was added. In the control tube 2 µl of ultrapure water was added instead of DNA. Total reaction volume was 20 µl. The reaction was amplified in a Thermal Cycler (TECHNE, UK).Polymerase chain reaction amplification was performed with the following protocol: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 50°C for 1 min, 72 °C for 1 min followed by a final extension step of 72°C for 5 min. The size of the PCR products was determined by agarose gel electrophoresis using appropriate size markers. DNA sequencing was performed using ABI 3130; Applied Biosystems according to the manufacturer instructions. Sequence similarity searches were realized using the online sequence analysis resources Blast. Phylogenetic relationships based on 5,8S rRNA gene sequence analysis of bacterial strain Abs3b was realized by the free program (Dereeper *et al.,* 2008). Evolutionary distances were calculated using the method of Jukes and Cantor.

Kinetics of lipase production

To follow the kinetics of lipase production, the three yeasts are cultured at 30 ° C in YPG medium supplemented by olive oil and Tween 80. We took aliquots of these cultures at different times and we have quantified the total of oleic acid by spectrophotometer at 715 nm and at the same time we

determined the biomass of yeasts at 600 nm. Oleic acid is used as the standard range.

Results and discussions

Physical-chemical characterization of samples

The physical-chemical characterization is essential before any microbiological study. These physical-chemical factors play a role in Fundamental growth and reproduction of micro-organisms in their watery habitat thereby enhances their real lives. With the exception of phase 'lime', this has a basic pH, while the other phases of tanning show an acidic character, this because of the presence in water of different acids (organic acids of pigeon droppings, tannic acid, fatty acids...) which influence microbial biodiversity of these environments. The samples studied tanneries have high electrical conductivity. This character is mainly due to the high content of salts present in these salt pans tanning. Indeed, in addition to the natural wealth in minerals, the skins are kept with high salt concentrations in the artisanal tanneries

It is also important to note the low dissolved oxygen concentration and also a BOD5 / COD ranging between 0.1 and 0.05. These physical and chemical characters thus confer specific conditions for growth and develop micro-organisms from these environments. (Table.1)

Isolation and screening of lipolytic yeast strains

A total of 20 yeast isolates were obtained from pigeon droppings, bran and tanning. These yeast strains were examined for their lipolytic activity. In Table 2, the origin of the samples and the lipolytic activity of the yeast isolates are showed. The results indicated that 70% exhibit no activity, whereas only 30% of strains show variable lipase activity ranging from low to high. Four strains isolated from pigeon droppings exhibited high activity; however only one yeast strain isolates this biotope reveal no activity. Excepted two strains isolated from the bran biotope that show a low activity, the other 13 strains showed no lipase activity.

The yeast strains 7H, 8H and 9H growth on YPG medium addition on the

Table 1. Physical and chemical characteristics of traditional tanneries CHOUARA.

Phase of the tanning process	Lime	Pigeon droppings	Bran	Tanning
pH	10.49	5.40	2.74	3.16
Temperature	24.2	22.6	23.0	20.6
Dissolved oxygen (mg/l)	0.11	0.13	0.53	0.49
Conductivity (ms/cm)	28.8	19.23	24.4	12.53
BOD_5(mg-O2/l)	-	55	33	20
COD(mg-O2/l)	-	617	558	233

Table 2. Biotopes of yeasts and their potential lipases.

Biotope	Strain designation	Lipolytic activity
Pigeon dropping	7 H, 8 H, 9 H, LH1	+++
Pigeon dropping	LH8	-
Bran	LH2 and LH5	++
Bran	LH3, LH4, 23H, 24H, 26H, 27H, 28H and 29H	-
Tanning	LH6, LH7, LH9, LH10 and 51H	-

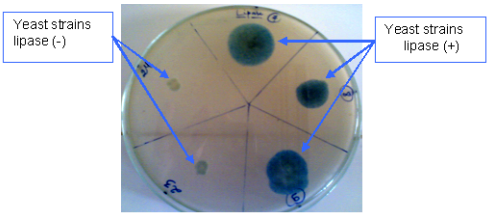

Figure 1. the blue halos confirming the lipase activity Yeasts 7H, 8H and 9H.

Victoria blue B. the revelation of the lipase activity was visualized by the development of a blue color around the colonies (Figure 1).

Molecular characterization of lipolytic isolates

Three yeasts that shows high lipase activity were sequenced (Figure 2). The comparison with GenBank database (EMBL) of the 5,8S rRNA gene sequences of these yeast isolate showing lipolytic activity indicated that the yeast strains (designed as 7H, 8H and 9H) could be identified it as *Trichosporon asahii*, *Trichosporon asteroids* and *Trichosporon coremiiforme* respectively. Phylogenetic analysis of these strains with the closest relatives is shown in Figure 3. The corresponding position of the three strains in its phylogenetic tree indicates that it is a new yeast strains. Other work has been isolated Trichosporon species from clinical specimens in Kuwait (Suhail *et al.*, 2009).

Stain	Sequence (5'P-3'OH)
7H	TCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTA TATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGTATTTTTACA AACAATGTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGA TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAAT TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAGCTTGCGCTCTCTGGTATTCC GGAGAGCATCGCTGTTTCAGTGTCATGAAATCTCAACCACTAGGGTTTCCTAATGG ATTGGATTTGGGCGTCTGCGATTTCTGATCGCTCGCCTTAAAAGAGTTAGCAAGTTT GACATTAATGTCTGGTGTAATAAGTTTCACTGGGTCCATTGTGTTGAAGCGTGCTTC TAATCGTCCGCAAGGACAATTACTTTGATCTGGCCTGAAATCAGGTAGGACATACC CGCTGAACTTAAGCATATCAATAAGCGG
8H	CGTAGGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTAATTGGCTTATAACTAT ATCCACTTACACCTGTGAACTGTTCTATTACTTGACGCAAGTCGAGTATTTTTACAA ACAATGTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGAT CTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCAGCTTGCGCTCTCTGGTATTCCG GAGAGCATGCCTGTTTCAGTGTCATGAAATCTCAACCACTAGGGTTTCCTAATGGAT TGGATTTGGGCGTCTGCGATCTCTGATCGCTCGCCTTAAAAGAGTTAGCAAGTTTGA CATTAATGTCTGGTGTAATAAGTTTCACTGGGTCCATTGTGTTGAAGCGTGCTTCTA ATCGTCCGCAAGGACAATTACTTTGACTCTGGCCTGAAATCA
9H	GGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTATATCCA CTTACACCTGTGAACTGTTCTATTACTTGACGCAAGTCGAGTATTTTTACAAACAAT GTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTG GCTCTCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGAA TTCAGTGAATCATCGAATCTTTGAACGCAGCTTGCGCTCTCTGGTATTCCGGAGAGC ATGCCTGTTTCAGTGTCATGAAATCTCAACCACTAGGGTTTCCTAATGGATTGGATT TGGGCGTCTGCGATCTCTGATCGCTCGCCTTAAAAGAGTAGCAAGTTTGACATTAAT GTCTGGTGTAATAAGTTTCACTGGGTCCATTGTGTTGAAGCGTGCTTCTAATCGTCC GCAAGGACAATTACTTTGACTCTGGCCTGAAATCAGGTAGGACTACCCGCTGAACT TAAGCATATCAATAAGCG

Figure 2. Sequences of 5.8S RNA for Yeasts 7H, 8H and 9H.

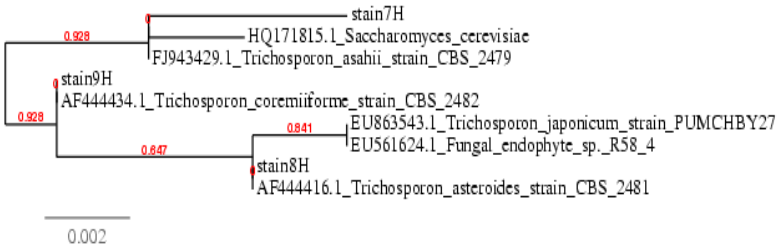

Figure 3. Phylogenetic tree (the branch length is proportional to the number of substitutions per site).

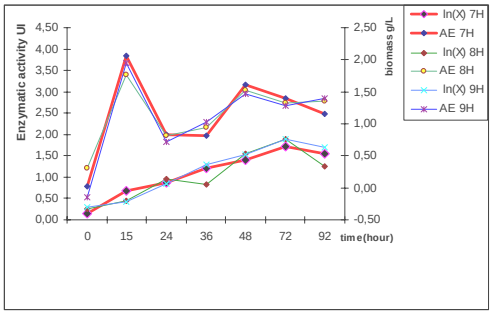

Figure 4. Kinetics of lipase activity for the yeasts 7H, 8H and 9H (the three top curves in Figure) and biomass evolution is represented by ln(X) (the three curves in the bottom of Figure).

Kinetics of lipase production and biomass of yeasts

The kinetics of lipase activity of the three yeasts 7H, 8H and 9H combined with the evolution of the biomass revealed two major phases of production of the enzyme. A first peak appears at 15 hours of culturing and coincides with the beginning of the exponential growth of yeasts. A second increase in production of lipase is manifested earlier than 75 hours of being cultivated. Maximum lipase production is done in two different times, in our culture conditions this result suggests that there are two different lipases, or one lipase that will only change its primary structure. These results are consistent with those obtained by Sayari *et al.* (2005) which unveiled on Rhizopus oryzae lipase is produced and ROL32 R0L29 which differ only by cleavage of the N-terminal fragment from ROL 29. The possibility of production of two lipase remains as valid as Chen *et al.* (1994) have purified two extracellular lipase from *Trichosporon fermentans*: Lip I and Lip II with different molecular weight stability at pH range of 4.0 - 8.0 in 30 ° C and the same capacity for hydrolysis of triglyceride in position1, 2 and 3.

Several studies have shown lipases produced by various yests (Vakhlu and Avneet, 2006), *Candida rugosa*, *Geotrichum candidum*, *Saccharomycopsis lipolytica*, *Candida lipolytica*, *Candida paralipolytica* (Redondo *et al.*, 1995, Tsujisaka *et al.*, 1973; Sugiura *et al.*, 1976; Ota *et al.*, 1982; Ota and Yamada, 1966). In several studies it was shown that Genus *Trichosporon* is another yeast studied for lipase production, *Trichosporon fermentans*, *Trichosporon asteroids*, *Trichosporon aszhii*, (Chen *et al.*, 1993, Dharmsthiti and Ammaranond 1997, Kumar *et al.*, 2009, Dharmsthiti and Ammaranond, 1997; Juliana *et al.*, 2007). To our knowledge this is the first work that shows the production of lipase by *Trichosporon coremiiforme*.

In conclusion, this work shows for the first time a lipase production by *Trichosporon coremiiforme*. This yeast is isolated from CHOUARA traditional tanneries of fez medina.

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