Characterization of Lipolytic Microorganisms From Traditional Tanneries of Fez (Morocco)

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Abstract— This study led on the traditional tanneries of Fes aims at the characterization of the skins treatments stage. The most important of which are the one dropping of pigeons which by lipolytic effect of the microorganisms, would be at the origin of the skins degreasing and softening. The high lighting of the lipolytic microbial potential would be nil doubt mattering not only for the adoption of a method of non-polluting clean tanning of the environment but also for the unpublished biotechnological progress in the field of lipases application. The analysis of data collected further to the physic-chemical and microbiological characterizations in the various stages of tanning revealed the extreme character and the microbial wealth in this site. The pigeon's droppings stage revealed three lipase producing yeasts. The kinetics of lipolytic activity for these yeasts with the biomass showed an important production of lipase in two phases suggesting the hypothesis of two synthesis lipases by the same yeasts.

Index terms—: traditional tanneries, physico-chemical, characterization, yeast, lipase.

1. Introduction

The Moroccan leather industry retains its place as one of the top producers on the international market, despite the wave of economic recession suffered by most industrialized countries. Moroccan exports leather and its derivatives are 326.6 million EUR in 2013, a figure growing thanks to a skilled workforce, knowledge how ancestral, a material first quality, geographical proximity, compared to the major markets of flow.

Today, this key sector faces a new challenge: minimize its impact on the environment, by holding its methods of production with a new method, detecting its inefficiencies and realizing that can also make additional profits, by reducing the generation of pollution. Traditional tanneries are pioneers and can serve as a model in a process of environmentally friendly "green" tanning.

Tannery "Chouara" in Fez medina is one of the emblems. It is the senior of the three tanneries of the ancient city that remain faithful to the tradition, in principle less polluting. It uses natural materials to produce leather. It is lime, of pigeon droppings, bark of grenadine and wheat bran. This traditional practice, declining, as modern tanneries prefer chemicals: more efficient, but much more polluting substances.

The traditional tannery has mainly four phases. The craftsmen tanners qualify phase of skin treatment by the pigeon droppings as the rate-determining step.

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Wild pigeon droppings contain micro-organisms that would be able to eliminate the fat from the skin. It is actually a potential activity lipase by these microorganisms. This observation suggests that this stage of bating using pigeon droppings is a key for the skins traditional tanning [1].

Lipases can perform not only hydrolysis reactions, but also synthetic reactions such as esterification, acetolysis and Alcoholysis. These reactions can take place as well in that organic aqueous media and with broad substrate specificity that can catalyse a variety of biotransformations chemo-, regio – and enantioselective [2].

It is in the context of the preservation of the water resources and the reconquest of the aquatic environment quality as well as for the development of innovative and eco-friendly in the tanning field, that fits the present work focuses on the valorization of microbial resources by use of their enzymes with a view to adopt a tannery traditional cleaner. Indeed, our goal is based on the isolation of lipolytic micro-organisms in stage of pigeon droppings.

Thus we did a physic-chemical characterization of the different phases of tanning, we then realized the isolation of microorganisms by a culture and we studied their lipolytic capabilities. Secondly we

have characterized important lipolytic potential yeast and the kinetics of lipase activity was studied. Yeasts which are isolated would be subsequently used in tanning traditional to improve step of cleaning skins and also in the bioremediation of effluents from the Sebou River.

II. MATERIAL AND METHOD

2.1 Study Site

"Chouara" tanneries are established since the middle ages. It is one of the most spectacular places located in the middle of the *Fez el Bali* medina in a district of the tanners. The crafts manship of leather takes place in four main phases.

1. Step of lime baths;
2. Step of pigeon droppings ditches;
3. Step of barn;
4. Step of Tanning.

These different stages involved during a complete tannery and the duration of each stage varies depending on the season and the origin of skin tanner (bovine, ovine or caprine animals).

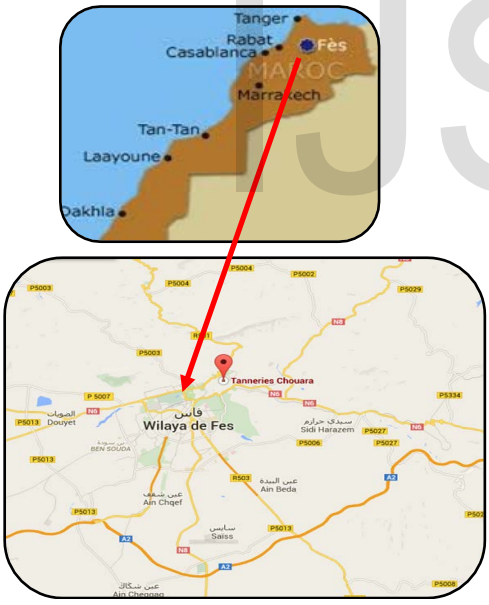

Figure 1: Geographical location of the traditional tanneries "Chouara" of Fez

2.2 Physico-chemical characterization of tanning waters

2.2.1 Sampling

Two samples were conducted at the level of the basins of tanneries. The first sampling is conducted during the month of May while the second is done in the month of December. Samples are carried out in sterile bottles of one liter.

2.2.2 Parameters analyzed

The methods of analysis are those recommended by the standards [3; 4]. The temperature and pH are measured by a pH meter type "ORION" on-site concentrations of dissolved oxygen in different samples of water from the four stages of tanneries are determined by a portable Oximeter and a second determination is performed at the laboratory by the Winkler method. Electrical conductivity is measured by a conductivity Type "WTW model LF318/set". The study of the biological and chemical applications oxygen (BOD, COD) are respectively performed by experimental devices of type "Oxi-(IS6)" and "Hot Record fuse T5A".

2.3 Microbiological testing of the samples

Water samples were collected in sterile and transported glass vials in chilled (4 C°) cooler to the laboratory. Microbiological analysis is carried out by cultivation and isolation of microorganisms from waters of the tanneries. To do this, we used two culture mediums whose composition is contained in table1.

Luria – Bertoni (LB) for bacteria culture medium.
Yeast Peptone Glucose "YPG" for yeasts culture medium.

Table1: composition of the culture media used for the isolation of microorganisms populating the waters of tanning

Medium	Constituents	Quantity	T °C and Incubation time
LB Medium	Peptone	10 (g)	30 °C for 24h
	Yeast extract	5 (g)	
	NaCl	10 (g)	
	Agar	20 (g)	
	Distilled water	qsp/l	
YPG Medium	Peptone	20(g)	37 °C for 24h
	Yeast extract	10(g)	
	Glucose	20(g)	
	Agar	20(g)	
	Distilled water	qsp/l	

From water samples, we prepared dilutions from 10^{-1} to 10^{-4}. A volume of 100 µl of different dilutions is spread on each of the media in triplicate. The colonies of bacteria and yeasts that are developed are subject to multiple purifications by rambling to obtain pure microbial isolates. These microorganisms isolated from traditional tanneries of Fez have trained a Bank of bacteria and yeasts. Two copies of this harvest are stored in glycerol.

2.4 Evaluation of the lipolytic potential of isolated microorganisms

The highlight of the lipase activity is performed on culture medium at base of Rhodamine B.

This method involves the measurement of the fluorescence caused by fatty acids released by the

action of lipase on olive oil. A quantitative fluorescence due to lipase is based on the interaction of Rhodamine B with the fatty acids released during enzymatic hydrolysis of olive oil [5]. The medium of Rhodamine B is composed of olive oil (30 ml), Tween 80 (250ml),Rhodamine B (0.02% w/v) and distilled water (50 ml). 50 ml of this medium is mixed with450 ml of basal (YPG or LB) medium.
The final medium is adjusted to pH= 7. Lipase activity colonies develop an orange fluorescence at the exhibition of boxes of Petri dishes (350 nm) UV radiation.

2.5 Kinetics of lipase production associated with yeast biomass

The YPG culture medium is used as basal medium to allow yeast 7 H, 8 H and 9 H to produce lipase, crop circles are autoclaved, and yeasts are incubated at a temperature of 30 ˚C with shaking (150 rpm). During incubation, the determination of lipase activity is permitted per color channel according to the Protocol described by [6]. A mixture has been prepared: 2.5 ml of olive oil is emulsified in 50 milli-molars, buffer phosphate at pH = 7, we also add 20µl of $CaCl_2$ (0.02 molar) and 1 ml of supernatant of three yeast culture medium. The mixture was incubated at 60 C under agitation for half an hour. The reaction is stopped by 1 ml of acid hydrochloric (6N) and 5ml of heptanes. The oleic acid concentration is determined by measuring the absorbance of oleic acid in solution in heptanes at 715 nanometers. A unit of lipase activity is equivalent to 1 micromole of oleic acid released by ml of the culture medium and minute at 60 ° C.

2.6 Electrophoreses of lipases on polyacrylamide gel

2.6.1 Precipitation of lipases by ammonium sulfate

Cultivation of three yeasts for 15 hours is performed on YPG medium supplemented with olive oil; crops have suffered a centrifugation at 1000 rpm for 10 minutes. Supernatants are treated under agitation by 80% ammonium sulphate (52, 3g per 100ml of water) and then we proceeded to a second centrifugation at 1000 rpm for 20 minutes, the obtained pellets are solubilized in a buffer Tris – HCl (10mM) at pH = 8.0.

2.6.1. Preparation of solutions

√ **Buffer Tris – HCL 0.5 M, pH 6.8:** 6 g tris base is solubilized in 60 ml of distilled water, the pH is adjusted at 6.8 with HCl (1N) solution. Completed the volume to 100 ml with distilled water then kept this buffer at 4 ° C.

√ **Buffer Tris – HCL 1.5 M pH 8.8**: 18,15 g tris base is mixed with distilled water, the pH is adjusted to 8.8 with HCl (1N) solution. Completed the volume to 100ml with distilled water then kept this buffer at 4 ° C.

√ **Solution 10% SDS:** 1g of sodium deshydro-sulfate is solubilized in water distilled under agitation, then the volume is completed in 10 ml.

√ **Acrylamide /bis-acrylamide (30%):** 29 g of acrylamide and 1 g of bis-acrylamide are mixed in a volume of 100 ml of distilled water. The solution is filtered and kept at 4 ° C.

√ **Sample buffer:** this buffer is prepared by 4 ml of distilled water, 1 ml of solution Tris – HCl (0. 5M, pH = 6, 8), 0.8 ml of glycerol, 1.6 ml of solution SDS 10%, 0.4 ml of solution of β-mercaptoethanol and 0.2 ml of bromophenol blue (0.01%)

√ **Electrophoresis buffer**: 5g of SDS, 72g glycine and 15g of Tris-base are solubilized in 1 litre of distilled water. This solution is then diluted to 1/5 by distilled water.

√ **Dye and bleaching gel:** the dye is prepared by the blue of coomassie (0, 1%) mixed with40 ml of ethanol, 10 ml glacial acetic acid and 50 ml of distilled water. Bleach is a solution containing 25 ml of ethanol, 10 ml of acetic acid and 65 ml of distilled water.

2.6.2 Preparation of gels

√ **10% separating gel**: this gel contains 4 ml of distilled water, 3.3 ml of acrylamide at 30%,2.5 ml Tris – HCl (pH = 8, 8 1.5 M 100 µl SDS (10%), 100 µl ammonium persulfate (10%)). The mixture is prepared with agitation under proboscis to water for the degasification. 4 µl TEMED is added and the mixture is poured between the plates for the polymerization of gelelectrophoresis. To avoid the contact of the gel with the oxygen in the air we covered the gel by a volume of butanol.

√ **Pregel (Staking gel**): this pregel has 3.5 ml of distilled water, 0.83 ml of acrylamide at 30%,0.63 ml Tris – HCl (1.5 M, pH = 8, 8), 50 µl SDS (10%), 50 µl (10%) ammonium persulphate. The mixture is prepared with agitation under trunk water degasification. 4 µl TEMED is added .the mixture is poured (gel) between the plates of electrophoresis for the polymerization of the pregel. Once the polymerization is complete we have removed the comb to free wells, and we have filed 40 µl of a solution of deposit (20 µl of sample buffer and 20 µl of buffer of lipases). At the end of migration, the gel is treated by coomassie blue dye solution and then by the bleaching solution of acetic acid and ethanol.

III. RESULTS AND DISCUSSION

3.1 Physico-chemical characterization of the traditional tanneries

3.1.1 Physical parameters: Temperature, pH and electrical conductivity

Except lime step that shows an alkaline pH, the other three phases of tanning are significantly acidic pH. These biotopes acidic character then allow a selection of microorganisms that are developed, adapting positively to these conditions. The electrical conductivity has recorded very high values whose maximum culminates 29 mS/cm. This state of facts would be of strong loads in naturally occurring salts from skins and also conservation salts (commercial salt) used by tanners before proceeding with the actual tanning.

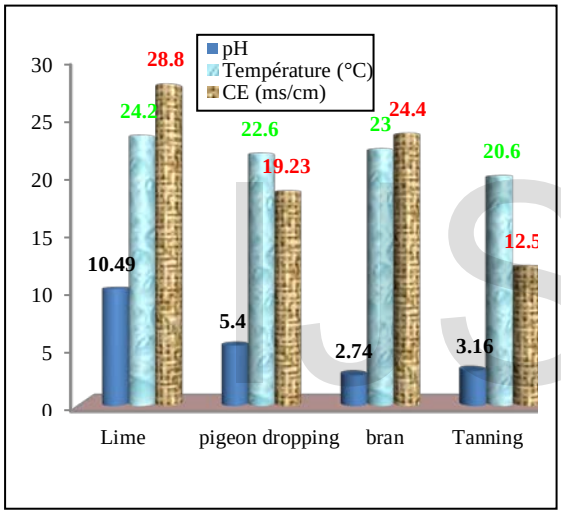

Figure 2: the fluctuations of these parameters

3.1.2 Chemical parameters: dissolved O_2, BOD_5, COD

The spatial pattern of the dissolved O_2 recorded very low values in all stages of tanning, which suggests the presence of anaerobic conditions. The measurement of BOD_5 and the COD of the three stages of tanning is presented in table 2.

It follows the important value of BOD_5 in phase of droppings of pigeon compared to the other two stages. This is explained by the presence of biodegradable organic matter-rich pigeon feces.

The COD values are more important than those of BOD_5 due to the presence in those graves of non-biodegradable chemicals (table 2). All these characteristics mark the extreme nature of the waters of tanneries.

Table2: Value of dissolved O_2, DBO_5 and COD of samples

	Lime	Pigeon droping	barn	Tanning
Dissolved O_2 (mg/l)	**0,11**	**0,13**	**0,53**	**0,49**
DBO_5(mg/L)	-	**50**	**31**	**0,1**
COD (mg/L)	-	**557,6**	**592,4**	**0,05**

3.2 Microbial biodiversity of tanneries waters

3.2.1 Culture and counts of microorganisms

a- Sampling of may

Table 3 demonstrates the enumeration of micro-organisms appeared in two culture media, the results show a wealth of microorganisms in the three phases of tanning process. Step dung of wild pigeon appeared also the busiest in microorganisms.

Table 3: Enumeration of micro-organisms colonizing the two culture media

	Medium LB			
	Without dilution	10^{-2}	10^{-4}	BM * (UFC /ml)
Lime	No colony	No colony	No colony	0
Pigeon droping	Microbial mats	478	914	$99,8 .10^5$
barn	Microbial mats	Microbial mats	528	$64,5.10^5$
Tanning	472	284	232	$68,4.10^5$
	Medium YPG			
	Without dilution	10^{-2}	10^{-4}	YB * (UFC /ml)
Lime	No colony	No colony	No colony	0
Pigeon droping	104	48	50	$3,5 .10^5$
barn	90	26	42	$4,5.10^5$
Tanning	20	3	No colony	$1,2.10^5$

BM: Bacterial biomass
YB: Yeast biomass

b- Sampling of December

A comprehensive analysis of the data in table 4 and figure 3, reveals that tanning basins workplaces are more loaded micro-organisms during the summer. This is the reason why during the winter, to treat in artisanal tanning skin under go long periods of stay during treatment. Note that Although the phase dung pigeon is marked by the greatest rate of microbial load, which will lead us to explore a new track research that could answer several questions regarding the lipolytic enzymes.

Table 4 : Enumeration of micro-organisms colonizing the two culture media

	Medium LB			
	Without dilution	**10-2**	**10-5**	**BM * (UFC /ml)**
Lime	**No colony**	**No colony**	**No colony**	**0**
Pigeon droping	**1200**	**80**	**7**	**0,5 .105**
bran	**170**	**-**	**20**	**11.105**
Tanning	**Microbial mats**	**172**	**101**	**4.105**
	Medium YPG			
	Without dilution	**10-2**	**10-5**	**YB * (UFC /ml)**
Lime	**No colony**	**No colony**	**No colony**	**0**
Pigeon droping	**760**	**30**	**100**	**2 .105**
bran	**21**	**48**	**5**	**9.105**
Tanning	**Microbial mats**	**100**	**122**	**7.105**

BM: Bacterial biomass
YB: Yeast biomass

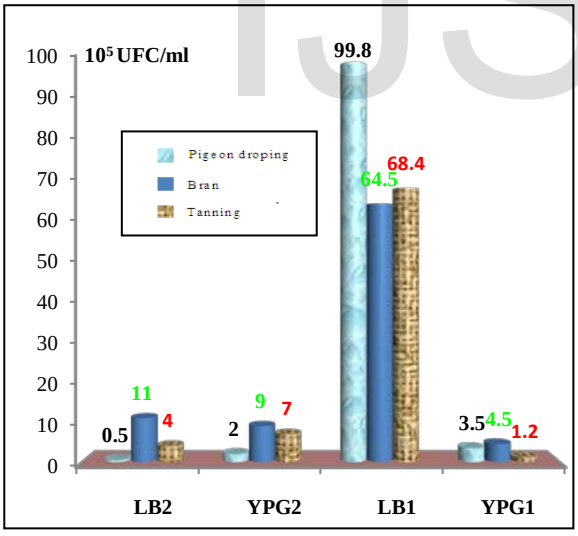

Figure 3: Variation of microbial load by culture medium and phase of tannery Withdrawals may (1) and December (2)

3.2.2 Purifications by shortages of isolates

The different colonies appeared on culture media were observed under the microscope and have subsequently suffered losses on medium LB (bacteria) and medium YPG (yeast). We were able to harvest a 65 bacteria and 29 yeasts Bank. Tables 5 show the inventory of bacteria and yeasts isolated and purified from tanneries and natural droping biotopes.

Table 5: Inventory of bacteria and yeasts isolated purified

		Pigeon droping	**bran**	**Tanning**
Sampling of May	Bacteria	20	17	10
	Yeasts	6	8	1

3.3 Characterization of the lipases activities from the microbial isolates

The enzymatic capability of lipase highlighted for samples is presented in table 6.

Table 6: enzymatic capabilities of type Lipase revealed in purified microorganisms

Biotopes	**Isolates**	**Codes isolats**	**Lipase**
Pigeon droping	**Bacteria**	11H	+
		32H	+
		38H	+
		39H	+
	Yeast	**7H**	+ + +
		8H	+ + +
		9H	+ + +
bran	**Bacteria**	14H	+
		19H	+
		21H	+
	yeast	23H	+

These results show that enzymatic expressions of the enzymes tested are manifested especially in phase pigeon droppings and phase "bran". The data indicate also that are especially yeast 7 H, 8 H and 9 H present in the phase dung pigeon massively manifesting the lipolytic activity.

3.3.1 Isolation of three lipolytic yeasts

The three yeasts 7 H, 8 H and 9 H are tested regarding their lipolytic power, using the blue Victoria method. These yeasts have developed good lipase activity. Figure 4 shows the revelation of this activity by blue halos development on the colonies.

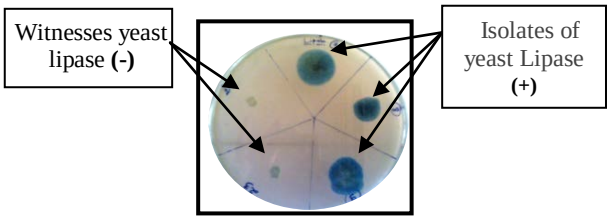

Figure 4: Revelation of lipase activity from yeasts: 7H, 8H and 9H

3.4 Kinetics of the lipase activity from yeast 7H, 8H and 9H

We conducted analysis of the kinetics of activities lipase in parallel to the biomass of yeast. The production of lipase for the three yeasts during the culture period revealed two important phases.

√ 15 hours of incubation phase which is marked by strong enzymatic activity of three yeasts, in particular that of yeast 7 H.
√ 72 hours incubation phase, where yeasts have also arisen another production increased by lipases. Between these two phases the lipase activities were discarded. Figure 5 shows the behavior of lipase (in solid lines) production combined with the evolution of the biomass in sticks of yeast 7 H, 8 H and 9 H

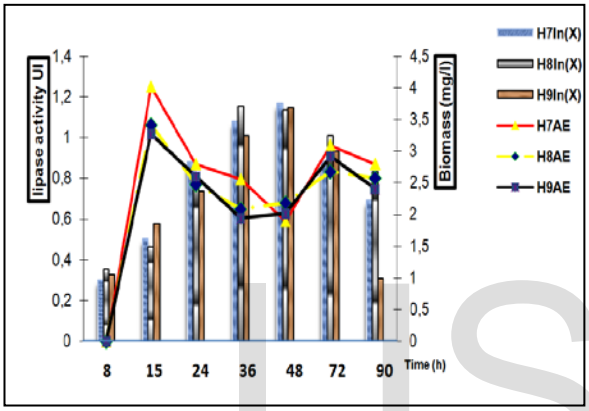

Figure 5: Kinetics of lipase activity associated with the biomass yeast

3.5.Electrophoresis of lipases on polyacrylamide gel

The literature suggests that the molecular weight of most lipases of yeasts varies from 45 to 60 KDa. The bands of lipase on SDS-page for our three yeast indicate that their molecular weights would be of the order of 55 KDa. This is then in accordance with the results of [7] who have purified two lipases I and II from *Trichosporon fermentans* WU - C12 and showed that (SDS-page) of these lipases, molecular weights 53 KDa and 55 KDa respectively.

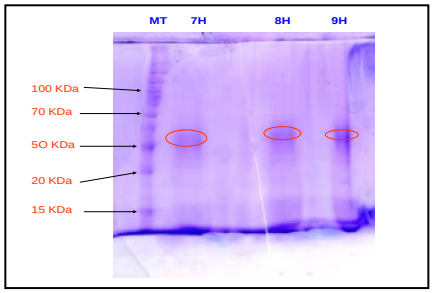

Figure 6 : SDS-PAGE of three yeast 7H, 8H and 9H

The physico-chemical analysis of the tanning phases indicates the character alkaline and barren limestone step. The other three phases of tanning (pigeon droppings, bran, tannins) revealed instead of acidic pH and low dissolved oxygen concentrations.
Acidity (abiotic parameter) would have influenced the spatial distributions of the total microflora at the level of the last three steps. The phase dung shows the largest biomass in bacteria and yeasts. This could be linked to load fat skins as carbon source used for metabolism of microorganisms.
[8] have highlighted the effect of low pH on microbial exploitation of body fat metabolism. It is then retention of fatty acids and Ions H^+ in the environment through the Krebs cycle. The adaptation of bacteria to pre-culture with oleic acid as carbon source induces their enzyme equipment activation and induction of a metabolic alternative.
Cells synthesize specific enzymes and not specific for the hydrolysis of fats and the increase of biomass is in relation to catabolism fats (organic fractions) [9].
OCD allowances rates increase from phase dung until step tannin which is not consistent with the net decrease of biomass. This is to the low acidity of the bran and tannins treatments pits (pH respectively 2.74 and 3.16).
It is also important to note that the concentration of (not-biodegradable) products used in final phase of tanning is against growth and microbial development.
These fluctuations of microbial biomass in different stages of tanneries are in concordance with work [1] on bacterial diversity of the Fez tanneries.
Three yeast screened remarkable phase of manure to activities lipases were kinetics that unveils two major lipolytic periods. The first phase after 15 hours of culture coincided with the exponential phase of growth of yeasts and the second period is determined towards the phase of decline.
An important expression of lipase two-step activity puts into question the adaptations of these yeasts. Production of two lipases hypothesis is consistent with the work of [10] on yeast of the genus Trichosporon. They then highlighted two lipases in *Trichosporon fermentans* production, lipase I stable at 40 C° for 30 minutes at pH = 5,5 and another lipase II with the same characteristics and stability of 50 C° temperature.
[10] also showed that lipase produced by yeast *Trichosporon asteroids* maintains stability at temperatures around 70 C° and pH between 3 and 10.
[11] have shown that strain *Rhizopus oryzae* lipase "ROL32" and a second shorter form "ROL29" lipase. ROL29 differs from ROL32 by the lack of a fragment of 21 acid residues amino side N-terminal of the enzyme.

VI. CONCLUSION

Microbial resources represent natural and real assets if their operation is conducted under harsh environmental conditions and the exploitation of their potential towards targeted applications. The exploitation of microbial resources from the traditional tanneries "CHOUARA" of Fez-medina could be a good example in biotechnology applications. Isolation by culture of microorganisms from this site has demonstrated important microbial richness and interesting lipolytic activities. We collected a Bank of microorganisms that may be important for other subsequent studies. We also noted that on different pits, there are temporal variations and fluctuations in wealth of bacteria and yeasts. Highlighting of lipase activities showed significant expressions, particularly in three isolated yeasts. The study of kinetics of lipase production indicated the remarkable lipolytic ability of yeasts which is favour of a realization and a layout values of their applications in tanneries.

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