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ORIGINAL RESEARCH PAPER

ISOLATION AND IDENTIFICATION OF LIPASE-PRODUCING FUNGI FROM LOCAL OLIVE OIL MANUFACTURE IN EAST OF ALGERIA

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Abstract: The main objective of this work was primary screening and isolation of lipase-producing microorganisms from oil-mill waste. For the screening of fungal strains with lipolytic activity, we employed a sensitive agar plate method, using a medium supplemented with $CaCl_2$ and Tween 80. Another Tributyrin lipase activity was detected from clearing zones due to the hydrolysis of the triacylglycerols. The evolution of biomass and enzyme production has been assayed. A quantitative analysis of lipase activity was performed by the titration method using olive oil as a substrate supplemented with glucose or Tween 80. We have isolated some lipolytic strains from oil-mill effluent. Three of them were found to be excellent lipase producers that were identified as *Penicillium sp, Aspergillus fumigatus* and *Aspergillus terreus*. Lipolytic activity and biomass were enhanced in the medium supplemented by glucose. Tween 80 is also considered as a best inducer at the concentration of 1 %. In this condition, these isolates showed maximum lipase production within 24 h; achieved (3.91 $IU \cdot mL^{-1} \pm 0.12$) for *Penicillium sp.*

Keywords: *fungal lipase, olive oil, screening, Tributyrin, Tween 80*

INTRODUCTION

Lipases (triacylglycerol hydrolases EC 3.1.1.3) form a large group of enzymes that catalyze the hydrolysis of a wide range of carboxyl esters to fatty acids, mono and diacylglycerols and glycerol [1, 2]. These are widely distributed in plants, animals and microbes, where their physiological role is to metabolize lipids. These are obtained either by extraction from animal or plant tissue or cultivation of microorganisms [2].
Lipases are produced by many microorganisms, including bacteria, fungi, yeasts and *Actinomyces*, although *Candida*, *Rhizomucor* and *Rhizopus sp*. stand out nowadays as sources of most commercially available enzyme preparations [3, 4].
Fungi are microorganisms preferred as producers industrial lipases, because they usually produce extracellular enzymes, which facilitates the extraction of the fermentation medium. In addition fungi enzymes are more stable and have more diverse properties compared to lipases from other sources [5]. Lipases have been widely used for biotechnological applications in dairy industry, oil processing, and production of surfactants and preparation of enantiomerically pure pharmaceuticals. Lipases have a wide range of applications in industry (food, cosmetics, detergents, leather, pharmaceutics), in biomedicine and in bioremediation of environments polluted with spills containing hydrocarbons, wastes from oil manufacturing or food processing (vegetable oils or animal fat from house wastes or restaurants) [6 – 8].

MATERIALS AND METHODS

Isolation of lipase producing microorganisms

Strains of lipolytic fungi were isolated from oil-mill effluent. Olive mill waste used in this study was obtained from local olive oil manufacture from region of *Guelma* (Algeria).
The samples were inoculated to the potato dextrose agar (PDA) supplemented with Gentamycin 0.1 % (v/v), than fungi were cultured at 30 °C in tubes and Petri dishes on Sabouraud-dextrose agar (Sigma-Aldrich) [9].

Strain conservation

Isolates were cultivated on PDA at 30 ± 1 °C until colonies covered approximately two-thirds of the area of the Petri dishes. Discs of 0.5 cm in diameter were collected from the actively growing regions of the colonies and kept in sterile distilled water, *p*H 6.5, at 4°C, as stock for future inoculations [10].
The fungus was maintained routinely on 4 % PDA plates and the long-term stock was kept at -80 °C as a spore suspension in 15 % glycerol [11].

Preliminary selection of strains for lipase activity

The first selection of lipase-producing strains was the basis of the magnitude of the diffusion zone of the enzyme (diameter, cm) secreted into the agar medium by growing colonies [12]. Culture plates inoculated with strains were incubated at 30 °C for 72 h,

the radius (r) of the colonies and the radius (R) of the clear hydrolytic halos around them were measured [13]. The isolates were selected for further studies based on the largest zone of lipolysis.

Tributyrin agar plates

The preliminary lipolytic screening was carried out according to the methodology described by *Torres et al.* with modifications. The medium used was composed of (in $g \cdot L^{-1}$): mycological peptone (5.0), yeast extract (3.0), agar (20.0), and tributyrin (*glycerin tributyrat*) (Sigma-Aldrich) added to give a final concentration of 0.1 % (v/v), pH 6.0. After autoclaving the medium (121 °C, 20 min), the mixture was emulsified by vigorous shaking for 15 min [14] and after cooling to approximately 60 ºC, the medium was transferred to Petri dishes and rapidly cooled [15, 16].

The microorganisms were incubated at 30 °C. Lipolytic activity was identified on the plates as a transparent halo around the colonies after 7 days of incubation [3].

The development of a clear zone is an indication of lipolytic activity, and its area is a measure of the extent of activity [17]. Lipase production is indicated by the formation of clear halos around the colonies grown on agar plates containing tributyrin [18].

Tween 80 agar plates

Screening of lipase activity was carried out using the modified method of Abed Samad et *al*. The medium used for screening has the following composition in ($g \cdot L^{-1}$): NaCl 1, Tween 80 10, Red of methyl 0.1, Agar 20, Distilled water 1000 mL [19]. Culture filtrates were spotted onto wells of agar plates containing Tween 80**.** The formation of opalescence surrounding the fungal colony was recorded as positive for lipase production [20].

Assay of lipase activity

Growth media

For determination of lipase activity, the test fungal strain was grown on basal medium contained (in $g \cdot L^{-1}$): NaH_2PO_4 12, $MgSO_4 \cdot 7H_2O$ 0.3, KH_2PO_4 2, $CaCl_2$ 0.25, $(NH_4)_2SO_4$ at 1 % and olive oil at 2 % were used as nitrogen and carbon sources. The initial *p*H was adjusted to 6 for yeast and fungi [21]. Lipase production was performed in 250 mL Erlenmeyer flasks containing each 50 mL of the following culture medium, the flasks were incubated at 30 °C in a rotary shaker (150 rpm). Every 24 h for 4 days optic density OD was measured at 600 nm by spectrophotometer (*SECOMAM)*, and 5 mL samples were collected, filtered through *Whatman* paper [22]**,** and the culture filtrates were used as the enzyme sources. The *p*H of the medium was also determined by a pH-meter (Inolab pH 7110 WTW).

Determination of lipolytic activity

Lipase activity was determined by the titration of free fatty acids released from olive oil hydrolysis with 0.05 M NaOH using emulsified oil as substrate [23 – 26]. Activity against olive oil was determined with substrate emulsion prepared in a Homogenizer (Dahian Scientific, Co Ltd) by mixing 40 mL of olive oil in 400 mL of a 2 % solution of gum Arabic from acacia tree Sigma-Aldrich Chemical Co (India) prepared in distilled water [27].

The assay mixture according to Hasan S. *et al.* with some modifications contained 1 mL of appropriately diluted enzyme was added to 4 mL of emulsion, and 5 mL of 10 mM phosphate buffer at *p*H 7 [23, 27]. Other assay mixture contained 1 mL of olive oil, 4.5 mL of 50 mM acetic acid *p*H 5.6, 0.5 mL of 0.1 M $CaCl_2$ and 1 mL of enzyme solution [1]. The mixture was incubated for 15 min at 30°C with continuous stirring 120 rpm. One unit of lipase was defined as the enzyme that liberated 1 µmol of fatty acid per minute at 30 °C, *p*H 7 [28]**.** The hydrolytic activities of the lipases were determined at *p*H 5 (citric buffer) [29].

Biomass determination

The biomass was determined by weighing the dry cell after freezing-dry [30]; Samples were filtered (0.45 pm), after filtration, the mycelium was washed with water and with acetone. Finally, the filters were dried at 105 °C or at 80°C to constant weight [31 – 33]**.** Values were the mean of three sets of experiments run simultaneously [33].

Partial identification of fungal isolates

Partial identification of fungal isolates was done by visual observation in Petri dish culture. For visual observation, the isolates grown in PDA were used. The mode of mycelial growth, color, odor and changes in medium color of each isolate were examined daily [26, 31].
All reagents are pure analiticaly.

Effect of glucose and Tween 80 on lipase activity

The addition of other carbon sources was tested. Cultivation was performed in minimal salts medium with olive oil supplemented with glucose (2 %), Tween 80 at 1 % and 2 %. The fungal isolates were grown in flasks with 50 mL of medium at 30 °C for 96 h with shaking at 120 rpm.

RESULTS AND DISCUSSION

Screening and isolation of lipolytic fungi

Some strains were isolated and identified as *Penicillium sp., Aspergillus fumigatus* and *Aspergillus terreus* were selected showing the best lipase activity. These fungi were isolated from olive mill waste in east of Algeria. The isolates were purified and checked for their lipolytic potential using the tributyrin and Tween agar method. They were cultured with shaking and tested for the lipolytic activity. These fungi were also described by Gopinath S.C.B *et al.* [17]. In the Tween method, formation of calcium crystals was observed. The tributyrin method shows a clear zone. Active zones are increasing with a period of incubation time and these zones can be measured.
As shown in Figure 1, the fungus *Penicillium sp.* was identified as a good lipase producer comparing to *Aspergillus fumigatus.* The halo of this strain was 3.4 cm, 4.5 cm respectively in 4 days and sixth days of incubation. The fungus *Aspergillus fumigatus* showed a good lipase producer when we use 1 mL of Tween 80 (3.3 cm). Corzo *et al.* reported that lipase activity in the medium without Tween 80 was significantly lower than the lipase activity in the medium with Tween 80 [37].

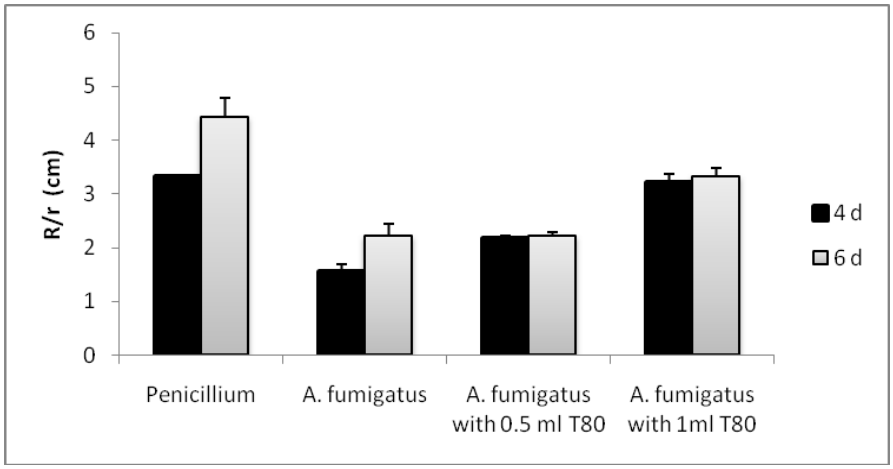


Figure 1. *Evaluation of the halos produced in medium agar plates containing Tween 80 on the fourth, and six days of growth of fungi*

Detection of lipolytic activity

Screening and isolation of microorganisms for lipase activity is most frequently carried out employing agar plates containing triglycerides or Tween 80. Lipase catalyzed hydrolysis gives rise either to clearing or opacity zones developed around colonies of lipolytic organisms [35].

The fungal strains which we had tested using agar medium supplemented with Tween 80 were lipase positive. By determination of the opacity areas developed around the colonies of the lipolytic microorganisms consisting of calcium [35].

Lipolytic productivity of strains was analyzed after comparison of the clear zone; Cihangir *et al*. observed that lipase activity as measured by the clearing of tributyrin depends on the time of cultivation [1].

The zones of hydrolysis produced by the Tributyrin breakdown after 72 to 96 hours of incubation period were due to the presence of lipase released by fungal isolates. Amongst these, the filamentous fungi are considered as an ideal source of lipases as they produce an extracellular enzyme [34]. The development of a clear zone of Tween-80 around the fungus was also an indication of lipolytic activity in agreement with Mukunda, S. *et al.* [20].

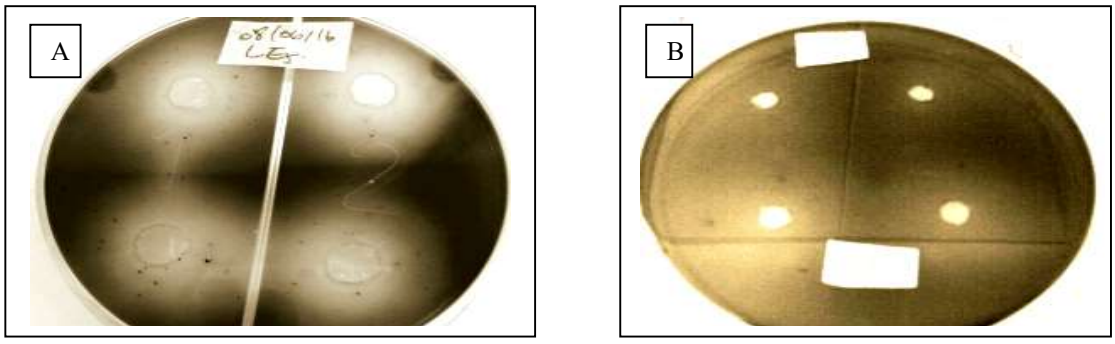


Figure 2. *The zones of hydrolysis after 6 days of incubation produced by Penicillium sp. spotted onto wells of agar plates containing Tween 80 (a) and zones of hydrolysis produced by A. terreus using tributyrin (b)*

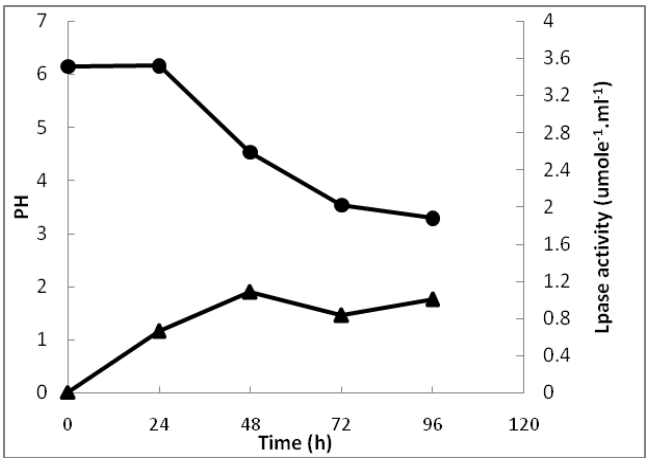


Figure 3. *Effect of incubation time on lipase activity of Aspergillus fumigatus (-▲-) and pH of culture medium (-●-)*

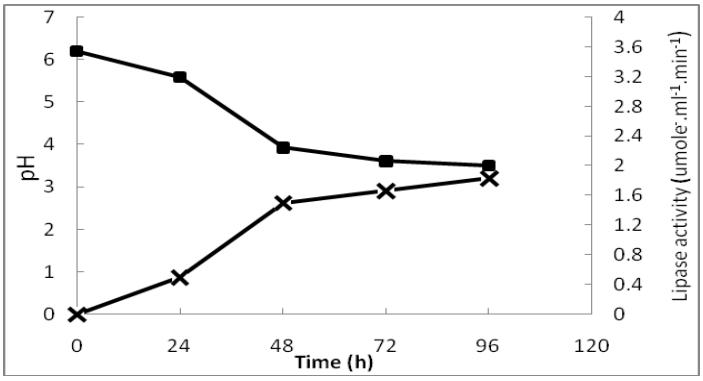


Figure 4. *Effect of incubation time on lipase activity of Aspergillus terreus (-X-) and pH of culture medium (-■-)*

As shown in Figures 3 and 4, the production of extracellular lipase increased steadily with cultivation time. It was demonstrated that the lipase activity is induced by the presence of lipid substrates in the medium as olive oil. This result confirms those reported by Falony, G. *et al.* [21].

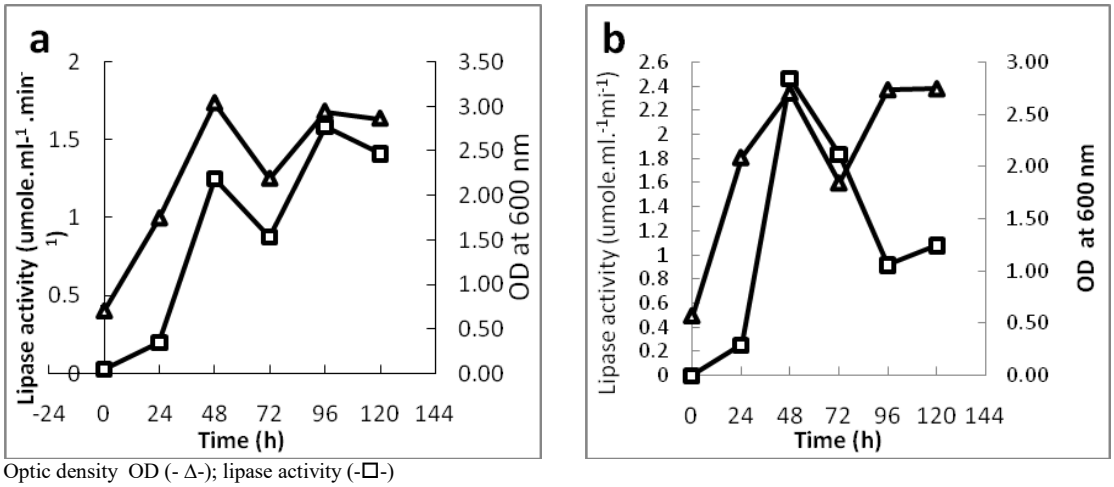
The final *p*H of both fermented mediums was noted towards the acidic side from the adjusted initial *p*H = 6.0; the final *p*H for *A. fumigatus* and *A. terreus* was 3.3 and 3.51, respectively, in contrast to result of *Naqvi S. H. et al.* they reported that the medium *p*H was increased [36]. According to Corzo G. *et al***,** the low *p*H of the culture might be the result of the production of organic acids such as oleic acid [37], which could affect lipase activity.

Highest lipase activity was found at *p*H 7. However, no lipolytic activity was detected in the culture of *p*H acid (*p*H 5) (data not shown) confirming results obtained by *Leow, T. C. et al*., who reported that none or low lipase activity was observed at *p*H below 6.0 [38].

Effect of glucose and Tween 80 on lipase activity

As shown in Figure 5, the isolate *A. fumigatus* exhibited maximum lipase production within 48 h, however, the biomass production decreased from 48 h to 72 h with lipase activity, practically the same, which agrees with *Colen G. et al.* [13]. The maximum

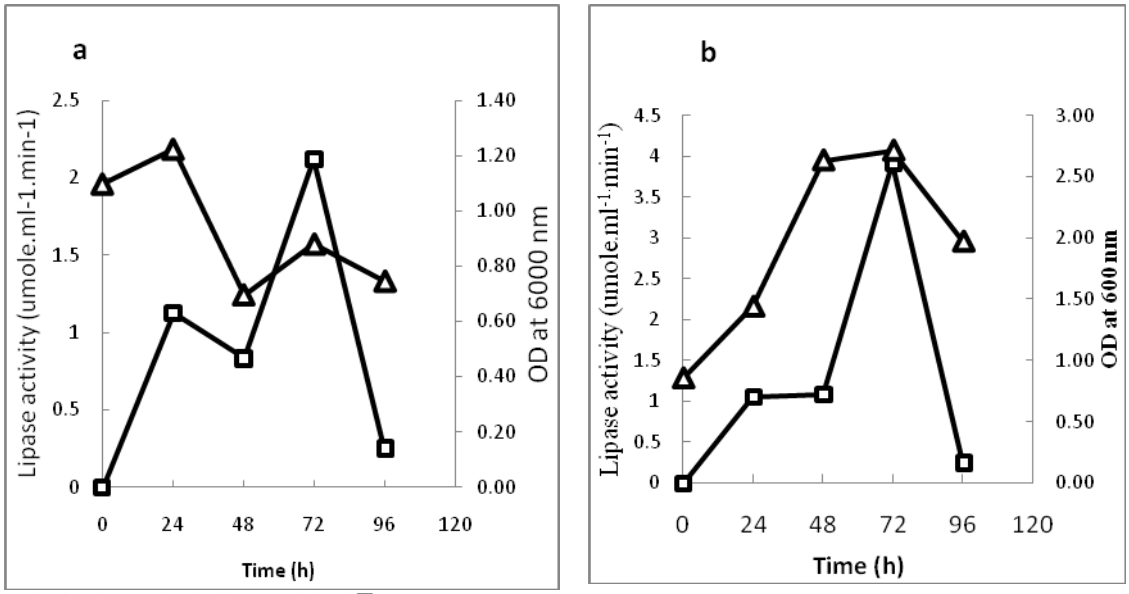
lipolytic activity at 48 h was found to be 2.5 IU·mL^{-1} in a medium containing 1% Tween 80, whereas the production of extracellular lipase decreased to 1.6 IU·mL^{-1} in a medium with 2 %.



Optic density OD (- Δ-); lipase activity (-□-)

Figure 5. *Extracellular lipase production by Aspergillus fumigatus isolates, in minimal mineral salts medium with olive oil and Tween 80 as inducer at 2 % (a), 1 % (b)*

Tween 80 in a concentration of 1 % was found to be the best inducer for the production of extracellular lipase and this is similar to the results of *Sathish Yadav K.N.* [39]. In contrast to some studies, it was determined that the concentrations of Tween 80 between 0.5 and 2 g·L^{-1} increased extracellular lipase activity without changes in the concentration of biomass [37].



Optic density OD (- Δ-); lipase activity (-□-)

Figure 6. *Extracellular lipase production by Penicillium sp. in minimal mineral salts medium with olive oil as inducer in absence of glucose (a) and in presence of glucose (b)*

The lipase activity of isolate is also stimulated by the addition of glucose to the production medium in agreement with results of *Ülker S et al.* [40].
The highest lipolytic activity was found in culture with glucose (around 3.91 IU·mL^{-1} ±0.12), which also gave different biomass levels; dry weight of strain *Penicillium sp.* reached to 794 ± 1.41 (mg) for 96 (h) as shown in Figure 6 (b), however lipolytic activity was found in culture without glucose around 2.12 IU·mL^{-1} Figure 6 (a), and the dry weight was 200 ± 0.71 (mg·mL^{-1}) (data not shown).
The reduction in lipase yield after an optimum period is probably due to the *p*H acid of medium.

CONCLUSIONS

Three strains were selected and identified as belonging to *Penicillium sp*, *Aspergillus fumigatus* and *Aspergillus terreus*.
It is concluded from the results of the present study that these isolates could be used as a good microbial source of lipase, and their production are induced by the presence of a lipid source as olive oil, Tween 80 at 1% and glucose.

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