Studii şi Cercetări Ştiinţifice 2019, **20** (1), pp. 011 – 019
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

EXTRACELLULAR LIPASE PRODUCTION BY *TRICHODERMA HARZIANUM* ISOLATED FROM OIL CONTAMINATED SOIL

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Received: March, 30, 2018
Accepted: January, 22, 2019

Abstract: The aim of this study was to investigate the ability of the filamentous fungus *Trichoderma harzianum* to secrete lipolytic activity and examined the influence of carbon on lipase production. A quantitative analysis of lipase activity was performed by the titration method using olive oil as a substrate. The isolated strain was cultivated in shaking flasks containing basal media and supplemented with olive oil 1 % (v / v) as lipid source. The effect of other carbon sources added to basal medium as lauric acid ester (Tween 80) and glucose was tested to improve enzyme production. Maximum biomass was produced at a concentration of (1906 $mg \cdot mL^{-1}$ ± 4.58) and (622.5 $mg \cdot mL^{-1}$ ± 9.19) in mineral medium supplemented with glucose (2 %) and Tween 80 respectively. However, the lipase activity was maximal for olive oil (1.58 $IU \cdot mL^{-1}$ min^{-1} ± 0.11). The result obtained in this study indicated that olive oil proved to be the best inducer and stimulated lipase production. Glucose and Tween 80 supplied as additional carbon sources have not enhanced the lipase production, whereas they only increased the biomass of *T. harzianum.*

Keywords: *fungal lipase, glucose, lipolytic-producing fungi, olive oil, screening, Trichoderma harzianum, Tween 80*

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INTRODUCTION

Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) are a class of enzymes that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface [1, 2]. The many applications of lipases include detergents, pharmaceuticals, cosmetics, leather processing, production of aliphatic acids, and in the treatment of domestic and industrial wastes [3, 4].
Microorganisms able to produce lipases can be found in several habitats, including wastes of vegetable oils and dairy product industries, soils contaminated with oils, seeds, and deteriorated food [5]. The soil has a great variety of microorganisms that can be isolated and evaluated for their potential as enzyme producers. Several methods can be used for microorganism screening based on the determination of the presence of extracellular lipases. The use of a solid medium with inducer substrates such as vegetable oils, standard triglycerides (Tributyrin), Tween 80, and dyes has been widely described in the literature. However, some of these substrates may not be adequate for lipase detection [6].
Among the microorganisms, fungi are recognized as one of the best lipase sources.
Fungal lipases are versatile in its enzymatic properties and substrate specificity, which make it very attractive for industrial applications [7]. Fungal enzymes are extracellular in nature and they can be extracted easily [8].
Lipases have been extensively studied because of their actual and potential applications in the detergent, oil and food industries. Recently, various strategies in the pharmaceutical and chemical industries have used lipases in the synthesis of pure drugs and agrochemicals [5].
Fungi are preferable lipase sources because fungal enzymes are usually excreted extracellularly, facilitating extraction from fermentation media. Filamentous fungi, especially those of genera *Rhizopus, Mucor, Geotrichum, Aspergillus, Fusarium* and *Penicillium* are widely used as sources of lipases [9].
During our screening of filamentous fungi which may produce extracellular lipase, we have isolated *Trichoderma harzianum* strain. The present study was to evaluate the ability of *T. harzianum* strain to hydrolyze olive oil. The influence of glucose and Tween 80 (lauric acid ester) on the lipase activity was checked.

MATERIALS AND METHODS

Isolation of lipase producing microorganism

Microorganism isolated from oil contaminated soil was isolated in our laboratory from soil samples collected from region of *Guelma* (Algeria). The fungal organisms present in the soil were isolated using soil dilution method [10]. The sample was inoculated to the Potato dextrose agar (PDA) supplemented with Gentamycin 0.1 % (v / v), then fungus was cultured at 30 °C in tubes and Petri dishes on Sabouraud-dextrose agar (Sigma-Aldrich) [11].
Isolate was cultivated on PDA at 30 ± 1 °C until colonies covered approximately two-thirds of the area of the Petri dishes, and kept in sterile distilled water at 6-8 °C, as stock for future inoculations [12].

Pure cultures of fungal strains were obtained by subculturing on PDA medium, and maintained at 4 °C [13].

Lipase production

For determination of lipase activity, the test fungal strain was grown on basal medium contained (in $g \cdot L^{-1}$): NaH_2PO_4 12 g, $MgSO_4 \cdot 7H_2O$; 0.3 g, KH_2PO_4 2 g, $CaCl_2$ 0.25 g, Ammonium sulphate $(NH_4)_2SO_4$ at 1 % and olive oil at 2% were used as nitrogen and carbon sources, The initial *p*H was adjusted to 6 [14]. The culture was inoculated in 250 mL Erlenmeyer flasks containing 50 mL production media. It was incubated at 30 °C under submerged fermentation conditions. The growth was measured in terms of OD at 600 nm (JENWAY 6300 spectrophotometer). The *p*H of the medium was also determined by a *p*H-meter. Samples were collected, filtered through *Whatman* paper**,** the cell free supernatant obtained was used as the enzyme source [12].

Determination of lipolytic activity

This assay was performed using olive oil as a substrate. Lipase activity was assayed by titrating the fatty acids liberated from olive oil with alkali [1].
Then the enzyme solution (1 mL) was added to 5 mL of substrate emulsion (mixing 1 mL of olive oil in 9 mL of a 2 % solution of gum Arabic from acacia tree Sigma-Aldrich Chemical Co (India) prepared in distilled water) and 4 mL of 0.1 M phosphate buffer, *p*H 7.0 (Na_2HPO_4-NaH_2PO_4). Samples were incubated for 15 min at 30 °C [3].
Enzyme activity was determined by titration of the fatty acid released with 50 mM NaOH. One activity unit of lipase was defined as the amount of enzyme, which released 1 µmol of fatty acid per minute under assay conditions [15].

Effect of carbon source additives on lipase production

For lipase production, the basal media containing olive oil 1 % (v / v) as lipid source was added. Glucose and Tween 80 were supplied as additional carbon sources at 1 % and 2 % (w / v).

Biomass determination

The biomass in growth media was detected according to dry weight. The growth media were filtered through pre-weighed filter paper and the biomass was then dried in an incubator at 30 °C for 24 h [1, 16].

Partial identification of fungal isolate

Partial identification of fungus isolate was done by visual observation in Petri dish culture and micro-morphological studies in slide culture. For visual observation, the isolate grown in PDA was used. The fungal isolate were observed for their growth characteristics (texture, pigmentation, form, spore formation) [17, 18].

Effects of pH and temperature on lipase activity

The *p*H effect on activity was studied by olive oil assay in a *p*H range of 4 - 8 using citrate-phosphate at 50 mM under standard assay conditions, as described above.
Temperature is a critical parameter that has to be controlled and varied from organism to organism for cell growth and enzyme production. In order to optimize the

temperature for maximum lipase production, basal medium was prepared and incubated at different temperatures ranging from 25 - 60 ºC at *p*H 7.0 using phosphate buffer (50 mM).
All used regents are pure analytical and were purchased from Sigma-Aldrich.

RESULTS AND DISCUSSIONS

Preliminary selection of strain for lipase activity

The strain *Trichoderma harzianum* isolated in our laboratory was screened for their lipase producing ability on solid agar. The microorganism plate screening on solid medium containing Tributyrin and Tween 80 show visible halos after 3 days of incubation.
Halo zones in medium containing Tween 80 (20 - 14 mm) were observed around circular wells with culture supernatant and in medium containing Tributyrin were (11 - 15 mm) (data not shown).

Lipase production

As shown in Figure 1, lipase production reached a maximum (1.58 $IU \cdot mL^{-1} \cdot min^{-1}$ ± 0.11) at 72 h in basal medium containing olive oil 1 % (v/v) as lipid source, confirming results obtained by [14], they reported that the production of lipase was more significant in culture medium added with lipids as the carbon source than in the culture medium without lipids. It was demonstrated that the lipase activity is induced by the presence of lipid substrates in the medium. The result in Figure 1 indicated also that there was decreasing in *p*H of fermented medium from 6.8 to 6.08 at 120 (h), this finding is in agreement with Corzo and Revah, they reported that the low *p*H of the culture might be the result of the production of organic acids such as oleic acid, which could affect lipase activity [20].

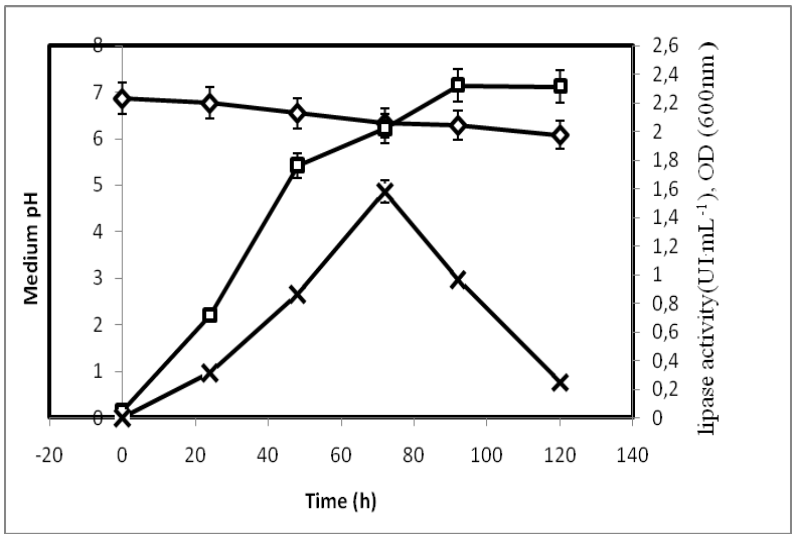

Figure 1. *Rate of production of fungal biomass OD (-□-), lipase activity (-X-) and medium pH (-◊-) during fermentation*

Figure 2 shows the rate of production of lipase activity, fungal biomass and medium pH change during fermentation in minimal medium supplemented by glucose (2 %) at 30 ± 1 °C for 120 h of incubation with shaking at 120 rpm.

Maximum growth rate was observed between 48 h and 72 h of fermentation, also lipolytic activity reached (0.96 $IU \cdot mL^{-1} \cdot min^{-1}$). After 72 h, there was a reduction in lipase activity and biomass.

According to Veerapagu et *al*. [21], reduction in the lipase production in the presence of sugars as carbon sources could be due to catabolic repression by readily available carbon sources in the medium.

It was also reported that further increase in the incubation period resulted decrease in the production of lipase. It might be due to the exhaustion of the nutrients and production of metabolic byproducts (inhibitors) in the fermenting medium. The accumulation of these byproducts resulted in decreased production of lipase by fungal strain [22].

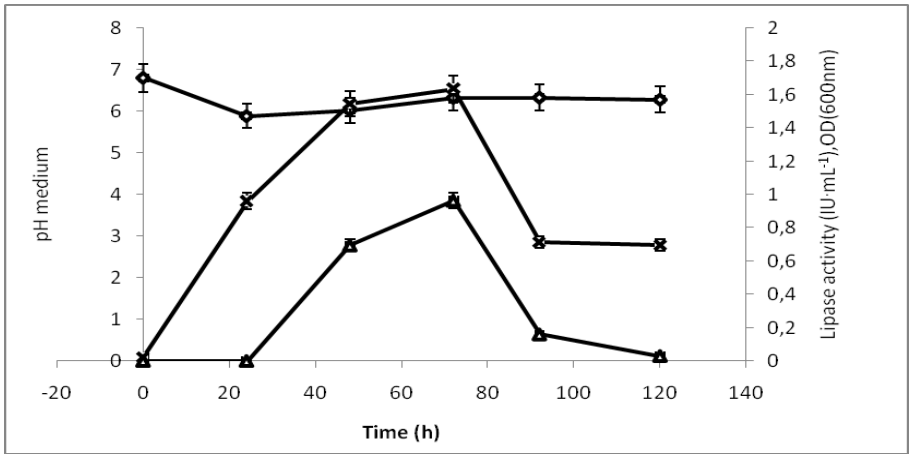

Figure 2. *Effect of incubation time on lipase activity ($IU \cdot mL^{-1} \cdot min^{-1}$) of Trichoderma (-Δ-), optic density OD (-X-), and pH of culture medium (-◊-) during fermentation*

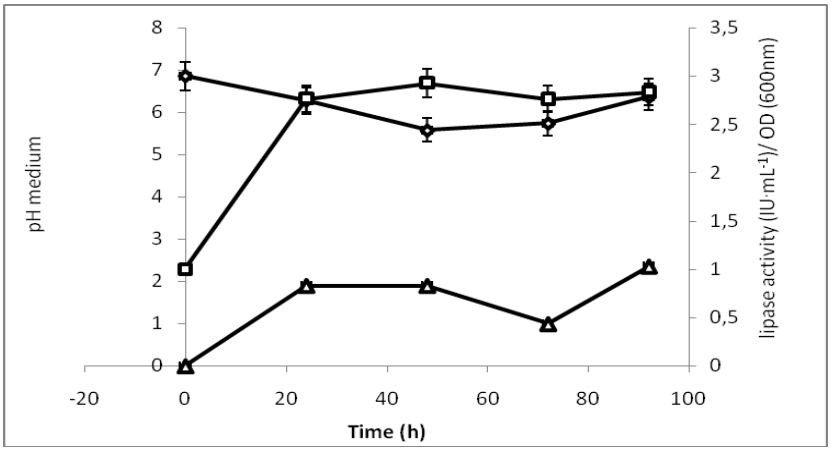

Figure 3. *Effect of incubation time on lipase activity of Trichoderma (-Δ-), pH of culture medium (-◊-),optic density OD(-□-), Cultivation was performed with mineral medium supplemented with olive oil and Tween 80 (1 %)*

The enzyme activity (1.0 IU·mL^{-1}·min^{-1}) was recorded in Figure 3; the microorganism was grown in flasks with 50 mL of medium at 30 °C for 96 h with shaking at 120 rpm. Adding Tween 80 (1 %) in the culture media did not increase the lipase production, however it increased biomass of strain. The result showed also that the *p*H of the medium decreased gradually from the initial *p*H of 6.7 to 4.4 in the exponential phase; then the *p*H increased to 5.5 during the stationary growth phase this report is in agreement with our findings [23].
The decrease in *p*H might be due to some organic acid production during enzyme production, and the increase in *p*H might be due to the production of free amino acids [24].
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 [20].

Effect of carbon source Additives on lipase production

Table 1 shown dry weight and Lipolytic activity of *T. harzianum* in different sources of carbon. Maximum lipase activity (1.58 U·mL) was recorded on day 4 of incubation of the culture at *p*H 6.8 and 30 °C, when medium was supplemented with olive oil in the absence of glucose and Tween 80. This result is in contrast with the report of a study carried out by Ülker et *al.* [16] and by Francis et *al*. [19]. In another study Lipase production is influenced by the type and concentration of carbon and nitrogen sources, as well as culture *p*H and temperature [24]. More biomass was achieved when glucose (2 %) was added to the medium (1906 mg·mL^{-1} ± 4.58), confirming results obtained by Bancerz et *al*. [26]. Similar results were obtained by Falony et *al*. [14], and Ramos-Sánchez et *al*. [27], they reported that sugar substrates only favor the growth of the microorganism but not the synthesis of lipase, whereas oleic acid, and olive oil enhance its synthesis.

Table 1. *Effect of carbon source additives on lipase production and biomass of Trichoderma harzianum*

Media	**Dry weight 72 h [mg·mL^{-1}]**	**Lipase activity [IU·mL^{-1}·min^{-1}]**
Mineral medium supplemented with olive oil (M1)	244 ± 1.41	1.58 ± 0.11
(M1) plus Tween 80(1 %)	622.5 ± 9.19	1 ± 0.04
(M1) plus glucose (1 %)	530.5 ± 0.71	0.96 ± 0.05
(M1) plus glucose (2 %)	1906 ± 4.58	0.45 ± 0.06

Effects of pH and temperature on lipase activity

The effect of temperature on lipase production is presented in Figure 4, maximum lipase production was observed at temperature of 30 ºC. Data obtained revealed that incubation at temperatures below and above 30 ºC decreased enzyme production.

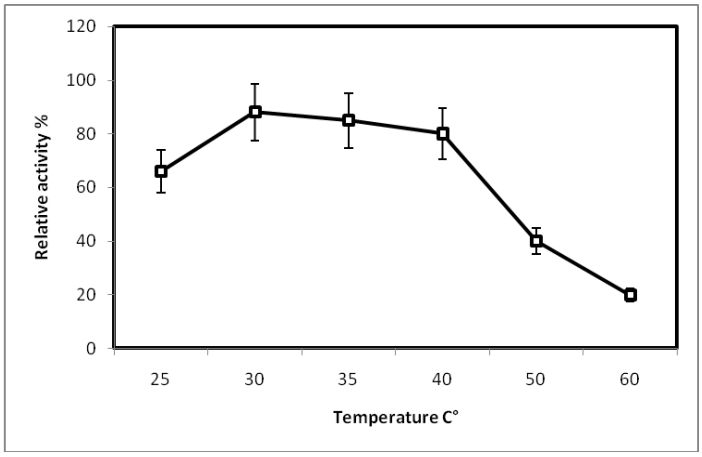

Figure 4. *Effect of temperature on lipase activity of Trichoderma harzianum (-□) Relative activity %*

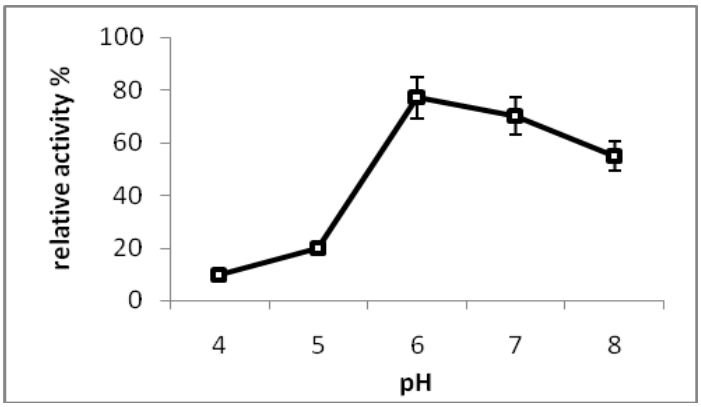

Figure 5. *Effect of pH on lipase activity of Trichoderma harzianum (-□-) Relative activity %*

Figure 5 shows that the activity of the enzyme increased from *p*H 5.0 to 6 and highest lipase activity was found at *p*H 6, which is in agreement with the finding of Ayinla et *al.*; they reported that most fungal cultures prefer a slightly acidic *p*H medium for growth and enzyme biosynthesis [17].
Lipase production decreased at alkaline and acidic *p*H of 8.0 and 4.0 respectively, 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 [28].

CONCLUSION

In this study, we identified a strain of *Trichoderma harzianum* which excretes an extracellular lipase. Secretion of lipolytic enzymes in submerged cultures of the strain this has been investigated; better lipase yields were obtained in minimal medium with oil olive as the sole carbon source.
Glucose and Tween 80 enhanced the growth of *T. harzianum* whereas they decreased the production of extracellular lipase by this strain.

REFERENCES

1. Cihangir, N., Sarikaya, E.: Investigation of lipase production by a new isolate of *Aspergillus sp*., *World Journal of Microbiology and Biotechnology*, **2004**, **20**, 193-197;
2. Kapoor, M., Gupta, M.N.: Lipase promiscuity and its biochemical applications, *Process Biochemistry*, **2012**, **47**, 555-569;
3. Hasan, F., Shah, A.A., Hameed, A.: Industrial applications of microbial lipases, *Enzyme and Microbial Technology*, **2006**, **39**, 235-251;
4. Qin, L-N, Cai F-R, Dong, X-R., Huang, Z-B, Tao Y., Huang, J-Z; Dong, Z-Y.: Improved production of heterologous lipase in *Trichoderma reesei* by RNAi mediated gene silencing of an endogenic highly expressed gene, *Bioresource Technology*, **2012**, **109**, 116-122;
5. Sharma, R., Chisti, Y., Banerjee, U.C.: Production, purification, characterization, application of lipases, *Biotechnology Advances*, **2001**, **19**, 627-662;
6. Griebeler, N., Polloni, A.E., Remonatto, D., Arbter, F., Vardanega, R., Cechet, J.L., Di Luccio, M., de Oliveira, D., Treichel, H., Cansian, L., Rigo, E., Ninow, J.L.: Isolation and Screening of Lipase-Producing Fungi with Hydrolytic Activity, *Food Bioprocess Technology*, **2011**, **4**, 578-586;
7. Singh, A.K., Mukhopadhyay, M.: Overview of Fungal Lipase: A Review, *Applied Biochemistry and Biotechnology*, **2012**, **166**, 486-520;
8. Mehta, A., Bodh, U., Gupta, R.: Fungal lipases: a review, *Journal of Biotech Res*earch, **2017**, **8**, 58-77;
9. Sumathy. R, Vijayalakshmi. M. Deecaraman. M.: Studies on Lipase production from fungal strains by different inducers at varied concentrations - A comparative study, *International Journal of Environmental Science*, **2012**, **3**, 1072-1078;
10. Thota, P., Bhogavalli P.K., Vallem, P.R., Srirangam, V.J.: Studies on optimization of extracellular lipase from potential fungal strain(s) isolated from oil contaminated soil, *Microbiology and Biotechnology Research*, **2012**, **2** (3), 418-425;
11. Yesilada, O., Sik, S., Sam, M.: Biodegradation of olive oil mill wastewater by *Coriolus versicolor* and *Funalia trogii:* effects of agitation, initial COD concentration, inoculum size and immobilization, *World Journal of Microbiology and Biotechnology*, **1998**, **14**, 37-42;
12. Schinke, C., Germani, J.C.: Screening Brazilian *Macrophomina phaseolina* isolates for alkaline lipases and other extracellular hydrolases, *International Microbiology*, **2012**, **15**, 1-7;
13. Toscano, L., Montero, G., Cervantes, L., Stoytcheva, M., Gochev, V., Beltrán, M.: Production and partial characterization of extracellular lipase from *Trichoderma harzianum* by solid-state fermentation, *Biotechnology and Biotechnological equipment*, **2013**, **27 (2)**, 3776-3781;
14. Falony, G., Armas, J.C., Dustet Mendoza, J.C., Martínez Hernández, J.L.: Production of Extracellular Lipase from *Aspergillus niger* by Solid-State Fermentation, *Food Technology and Biotechnology*, **2006**, **44**, 235-240;
15. Pabai, F.: Production, Purification, Characterization of selected microbial lipases and their application for interesterification of better fat, *Department of Food Science and Agricultural Chemistry,* McGill University, Montréal, Canada, Applied image, INC, **1997**, 179;
16. Ülker, S., Özel, A., Çolak, A., Alpay Karaoğlu, Ş.: Isolation, production, and characterization of an extracellular lipase from *Trichoderma harzianum* isolated from soil, *Turk Journal of Biology*, **2011**, **35**, 543-550;
17. Ayinla, Z.A., Ademakinwa, A.N., Agboola, F.K.: Studies on the optimization of lipase production by *Rhizopus sp. zac3* isolated from the contaminated soil of a palm oil processing shed, *Journal of Applied Biology and Biotechnology*, **2017**, **5** (02), 30-37;
18. Nwuche, C.O, Ogbonna, J.C.: Isolation of lipase producing fungi from palm oil mill effluent (POME) Dump Sites at Nsukka, *Brazilian Archives of Biology and Technology*, **2011**, **54**, 113-116;
19. Francis, S.I., Vivian, C. I.: Screening and Optimization of Process Parameters for the Production of Lipase in Submerged Fermentation by *Aspergillus carbonarius* (Bainer) IMI 366159, *Annual Research and Review in Biology*, **2014**, **4** (16), 2587-2602;
20. Corzo, G., Revah, S.: Production and characteristics of lipase from *Yarrowia lipolytica, Bioresource Technology*, **1999**, **70**, 173-180;
21. Veerapagu, M., Narayanan, S.D.A., Ponmurugan, K., Jeya, K.R.: Screening selection identification production and optimization of bacterial lipase from oil spilled soil, *Asian Journal of Pharmaceutical and Clinical Research*, **2013**, **3** (6), 62-67;

22. Iftikhar T., Hussain A.: Effect of nutrients on the extracellular lipase production by mutant strain of *Rhizopus oligosporus*, *Biotechnology*, **2002**, **1**, 15-20;
23. Rihani, A., Tichati, L., Soumati, B.: Isolation and identification of lipase-producing fungi from local olive oil manufacture in East of Algeria, *Scientific Study & Research Chemistry & Chemical Engineering, Biotechnology, Food Industry*, **2018, 19 (1)**: 13-22;
24. Rajendran, A., Thangavelu, V.: Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus MTCC 2233*, *Food Science and Technology*, **2009**, 1-26;
25. Silva, W.O.B., Mitidieri, S., Schrank, A. Vainstein, M.H.: Production and extraction of an extracellular lipase from the *entomopathogenic* fungus Metarhizium anisopliae, *Process Biochemistry*, **2005**, **40**, 321-326;
26. Bancerz, R., Ginalska, G., Fiedurek, J., Gromada, A.: Cultivation conditions and properties of extracellular crude lipase from the psychrotrophic fungus *Penicillium chrysogenum 9*, *Journal India Microbiology and Biotechnology*, **2005**, **32**, 253-260;
27. Ramos-Sánchez, L.B., Cujilema-Quitio, M.C., Julian-Ricardo, M.C., Cordova, J., Fickers, P.: Fungal Lipase Production by Solid-State Fermentation, *Journal Bioprocess and Biotechnology*, **2015**, **5**, 1-9;
28. Leow, T.C., Abd Rahman, R.N.Z.R., Basri, M., Salleh, A.B.: A thermoalkaliphilic lipase of *Geobacillus sp. T1*, *Extremophiles*, **2007**, **11**, 527-535.