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Original Article

Optimization of Extracellular Lipases Production by Fungal Strains Isolated from the Soil Enriched with the Oil of *Pistacia lentiscus*

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Abstract

Introduction: Lipases are enzymes that catalyze a large number of hydrolysis reactions leading to a vast diversity of industrial applications. Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their properties and ease of their production and extraction. The aim of this research work is the isolation of lipolytic fungal strains from the soil enriched with the oil of *Pistacia lentiscus* in the Annaba region of Algeria.

Materials and Methods: Fungi isolation was carried out on Potato Extract Agar (PDA) at 28 °C. Lipase activity was determined through the estimation of free fatty acids by titrimetry, optical density and final pH. The optimization of lipase production was achieved for several factors, namely, agitation, pH, temperature, biomass, carbon source, lipidic substrate, and time.

Results: Three strains were isolated and identified as *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp., with the latter exhibiting the highest lipolytic activity. The optimization indicated that these strains were able to hydrolyse different fatty substances with high activity under different conditions.

Conclusions: The obtained results show that pistachio oil was considered a novel inducer of the lipase-producing fungal strains.

Keywords: East Algeria, Molds, Lipase, Pistachio Oil, Production Conditions

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Introduction

Lipids are highly energetic, insoluble compounds consisting mainly of triglycerides (TG), which are triesters of glycerol and saturated, mono-unsaturated, or polyunsaturated fatty acids (FA). Lipolytic enzymes play a significant role in the metabolic degradation of lipids in different environments.[1] However, lipases, which are ubiquitous proteins, are among the most studied enzymes and are widely used in industry. They can be produced by different organisms, including animals, plants and microorganisms. Microbial lipases, particularly those from filamentous fungi and yeasts, are the preferred sources for industrial production.[2]

The significance of enzymes is continually increasing, particularly microbial lipases, which hold substantial industrial value due to their ability to catalyse diverse chemical reactions in both aqueous and non-aqueous media. The global lipase market is projected to exceed 797.7 million USD by 2025, growing at a compound annual growth rate of 6.2% from 2017 to 2025.[3] Industrial lipases are predominantly derived from microorganisms, as highlighted by Cai and Yang in 2023.[4] The microbial lipase market is estimated to be USD 425.0 million in 2018 and is projected to reach USD 590.2 million by 2023.[5] These enzymes find applications in various industries, including detergent formation, food and dairy product production, pharmaceuticals and medicine, biodiesel production, oleochemical industries, bioremediation, as well as the paper industry.[6-8]

Numerous researchers are actively exploring new sources to isolate microorganisms, particularly fungi, capable of producing novel lipases with specific characteristics for industrial applications.[9,3] Although many microbial lipases have been isolated and characterized, only some of them

have been commercially exploited. In order to cope with the growing industrial demands and overcome these shortcomings to replace traditional chemical catalysts, the preparation of new lipases with thermal/acid-base stability, regioselectivity, organic solvent tolerance, high activity and yield, and reusability through excavation and modification has become a hot research topic. Among them, lipase (EC. 3.1.1.3) is a very prominent biocatalyst, which has the ability to catalyze the hydrolysis and synthesis of ester compounds.[10] Microorganisms isolated from oil-based environmental samples were screened for their lipase-producing ability.[11]

Pistacia lentiscus L. is a tree belonging to the *Anacardiaceae* family. A medicinal species of it grows in all types of soil in sub-humid and semi-arid areas of Algeria.[12,13] The oil of this tree is commonly used in the treatment of various illnesses such as skin irritations, hair loss, gastric issues, respiratory problems of allergic origin, and stomach ulcers. Analysis of the total fatty acids composition revealed a predominance of unsaturated fatty acids, represented essentially by oleic and linoleic fatty acids. The triglyceride composition was mainly dominated by Palmitoyl-dioleylglycerol (POO) + Stearoyloleyl-linoleoylglycerol (SOL), Palmitoyl-oleyl-linoleoylglycerol (POL) + SLL + Palmitoyl-oleyl-palmitoylglycerol (PoOP), and Trioleylglycerol (OOO).

The presence of flavonoids and tannins in the essential oils of *P. lentiscus* L. has been revealed in different studies.[14] Furthermore, linoleic acid (C18:2) represents 20.95% to 23.77% of the total fatty acids, which are fatty substances that stimulate the production of lipase.[15] Although *P. lentiscus* is known for its antimicrobial potential, the question remains as to whether it could be a source of lipase-producing microorganisms at low concentrations. Therefore, this study aims to isolate lipase-producing fungal strains from soil enriched with *Pistacia lentiscus* oil for the first time.

Materials and Methods

Fungi Isolation

Fungal strains were isolated from soil enriched with *Pistacia lentiscus* oil in Annaba city (Northeast Algeria) using the standard dilution method on Potato Dextrose Agar (PDA) (Sigma-Aldrich). The isolated fungi were then purified on the same medium and stored at 4 °C.

Fungi Identification

Identification of the isolated fungi was conducted based on their macroscopic and microscopic morphology as described in the literature.[16,17]

Lipolytic Enzyme Production and Estimation

For the lipolytic activity determination, the strains were first inoculated with a 0.5 McFarland standard density in sterile Erlenmeyer flasks containing 200ml of the mineral medium. The medium consisted of 1000 ml distilled water, NaH_2PO_4 (12 g), $MgSO_4$.$7H_2O$ (0.3 g), KH_2PO_4 (2 g), $CaCO_3$ (0.25 g), $(NH_4)_2SO_4$ (1%, approx. 10 g), and olive oil 2%, (20 g) as nitrogen and carbon sources. Tween 80 (1%) was added to facilitate the diffusion of olive oil. [18, 19] The initial pH was adjusted to 7.0.[19] Then, the Erlenmeyer flasks were incubated for 72 h at 27 ± 2 °C in a shaker incubator. After fermentation, the liquid medium was filtered using 0.45 µm microfilters. Finally, the obtained clear filtrate represented the crude enzyme extract.

Evolution of pH and Fungal Growth during Fermentation

The fungal growth was estimated based on the optical density (OD) of the culture medium using a spectrophotometer at 600 nm.[20] The pH was measured using a pH meter.[18]

Estimation of the Lipolytic Activity

In this study, the lipase activity was determined using the quantitative titration method and the obtained enzymatic activities were expressed in International Units (IU). One unit (1 IU) of lipase activity is defined as the amount of enzyme that produces 1 µmol/min/ml of the product (free fatty acids).[21,22] Two drops of phenolphthalein were added to 5 g of the filtrate from the fermentation, along with 40 ml of ethanol and 9 ml of 2.25% Arabic gum (added to stabilize the emulsion) in 1000 ml of distilled water and vigorously homogenized. Subsequently, the medium was titrated with 0.1M KOH in ethanol until the solution turned pink.[14] Lipase activity was calculated following the method of Selva et al. (2008).[23]

Optimization of Lipase Production

The fermentation medium was used as a base, and the initial pH was varied between 3 to 8 to identify the optimal level. Simultaneously, the fermentation was conducted at different temperatures while maintaining the same initial pH to determine the optimal temperature for lipase production. In addition, the ability of the strains to degrade different fatty substrates, such as olive oil, pistachio oil and butter, with a concentration of 95% was also tested.

Statistical Analysis

All values were expressed as mean ± standard deviation (SD). Data were compared based on the mean values. Differences among the means of variety groups were tested using a Tukey–Kramer HSD (Software JMP version 7.0) with a significance level of 0.05.

Results

Isolation and Identification of Fungi

According to the morphological characteristics, three fungal species were isolated and identified as *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. (Figure 1A, B and C).

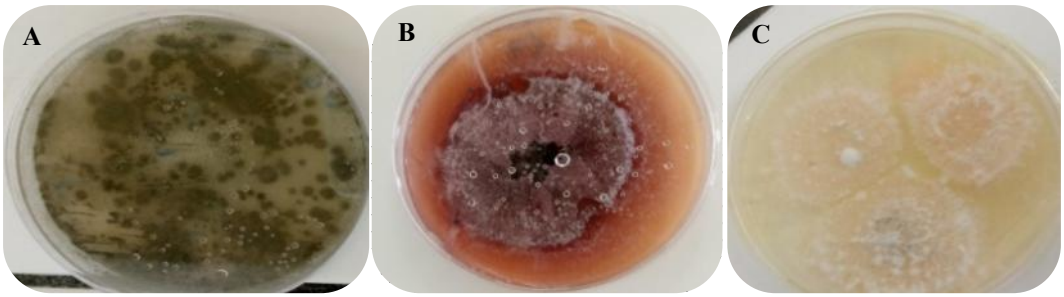


Figure 1. Macroscopic Aspects of the Isolated Strains Incubated on PDA Medium for 7 Days at 27 °C ± 2. **A)** *Aspergillus* sp. with mycelium olive green color; **B)** *Fusarium* sp. with mycelium with a reddish-pink color and a reddish pigment in the medium; **C)** *Penicillium* sp with snow-white mycelium in the form of dots.

Evolution of Fungal Growth and pH during Fermentation

Fungal growth

After 72 hours of incubation, the highest level of biomass production, as measured by OD, was observed in *Aspergillus* sp. (OD = 0.8 ± 0.17). In contrast, *Fusarium* sp. displayed the lowest level of biomass production, recording an OD of 0.18 (± 0.008), which was significantly different from both *Aspergillus* sp. and *Penicillium* sp. In addition, *Penicillium* sp. maintained an intermediate level of biomass, ranging from 0.58 (± 0.27) to 0.7 (± 0.31) throughout the incubation period, showing no significant difference with *Aspergillus* sp. (Figure 2A).

pH Values

The pH values resulting from the cultures of all fungal strains consistently decreased during fermentation. *Penicillium* sp. exhibited the lowest pH (3.2 ± 0.45), followed by *Aspergillus* sp. (3.8 ± 0.09), and *Fusarium* sp. (4.5 ± 0.31), which showed a statistically significant difference (Figure 2B).

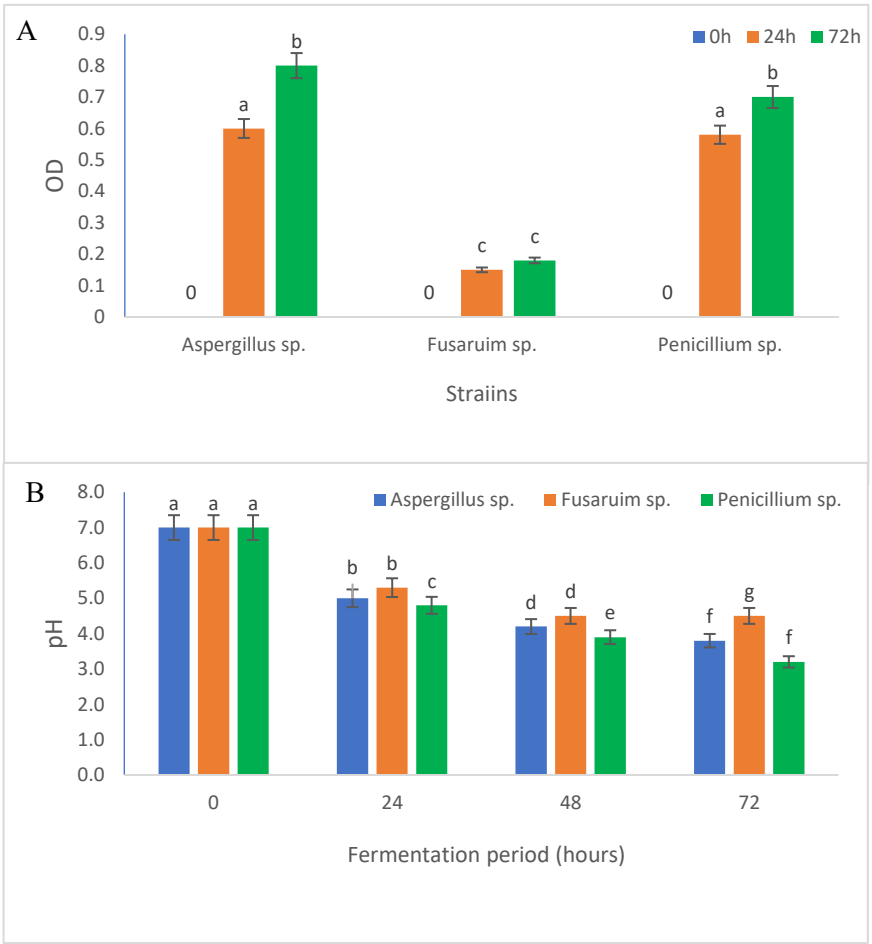


Figure 2. Evolution of Fungal Growth and pH during Fermentation. **A)** Optical density (OD) and **B)** pH during fermentation culture of the three isolated strains in mineral medium supplemented with olive oil. Values are mean ± SD. n = 3 in each group. Different letters indicate that samples are significantly different (p<0.05).

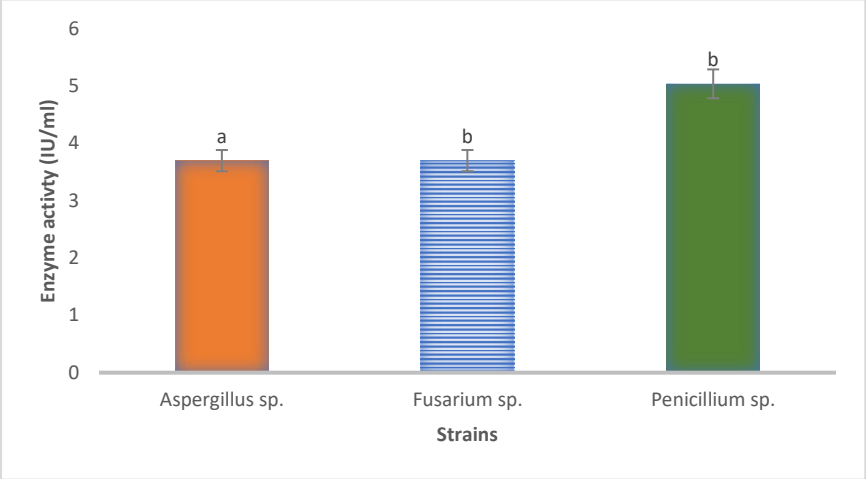


Figure 3. Activity of the Lipolytic Enzymes Production. Values are mean ± SD. n = 3 in each group. Different letters indicate that samples are significantly different (p<0.05).

Production and Optimization of Lipolytic Enzyme

As shown in Figure 3, *Penicillium* sp. exhibited the strongest ability to hydrolyze lipids with a lipolytic activity of 5.04 IU (± 0.08), highlighting its superiority in this aspect, followed closely by *Aspergillus* sp. and *Fusarium* sp, both with a lipolytic activity of 3.7 IU (± 0.12).

pH

The pH suitability range for all strains is limited between 5 and 9. *Penicillium* sp. exhibited significantly higher activity values than all other strains at pH 7.0 (Figure 4A**)**. There was no activity observed at pH 1 and 11 for any of strains.

Agitation

Penicillium sp. demonstrated a significant lipolytic activity (5.04 IU ± 0.34) with agitation, surpassing the activity observed without agitation (Figure 4B), comparable to *Aspergillus* sp. and *Fusarium* sp.

Biomass

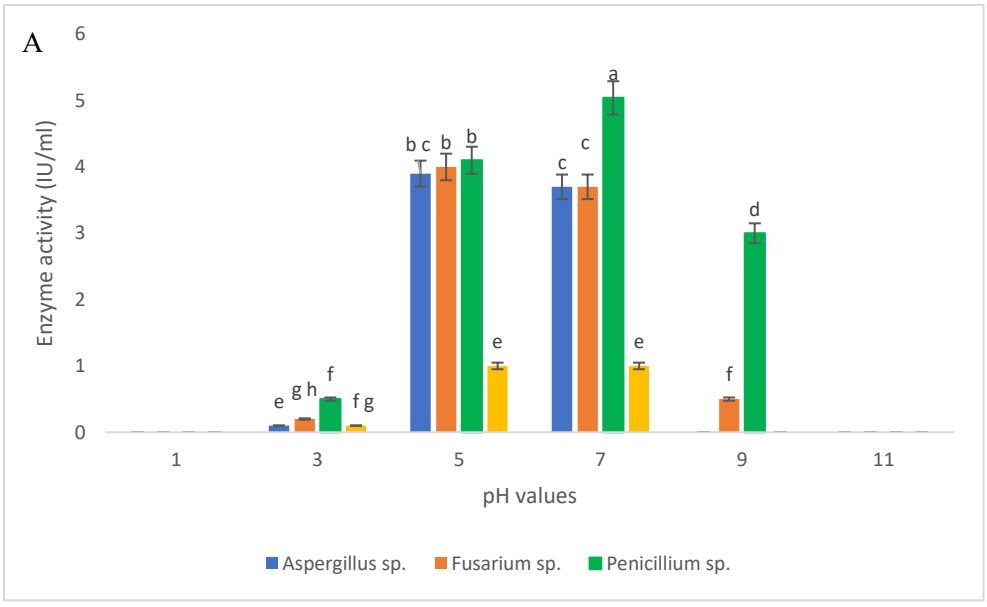
As depicted in Figure 4C, there was an inverse relationship observed between the lipolytic activity and the increasing fungal biomass, except for disks 3 and 4 where there are no significant differences.

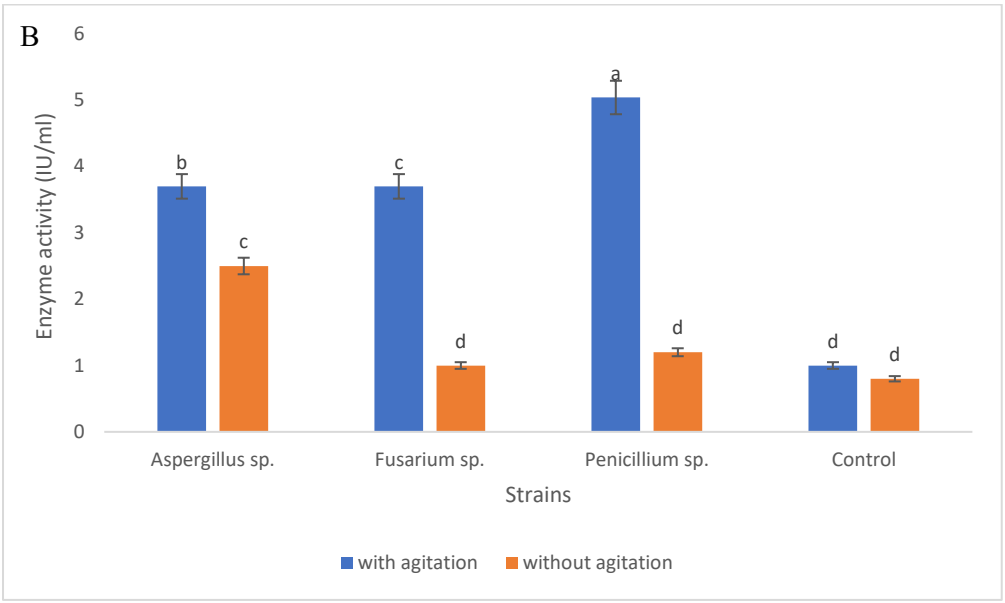
Temperature

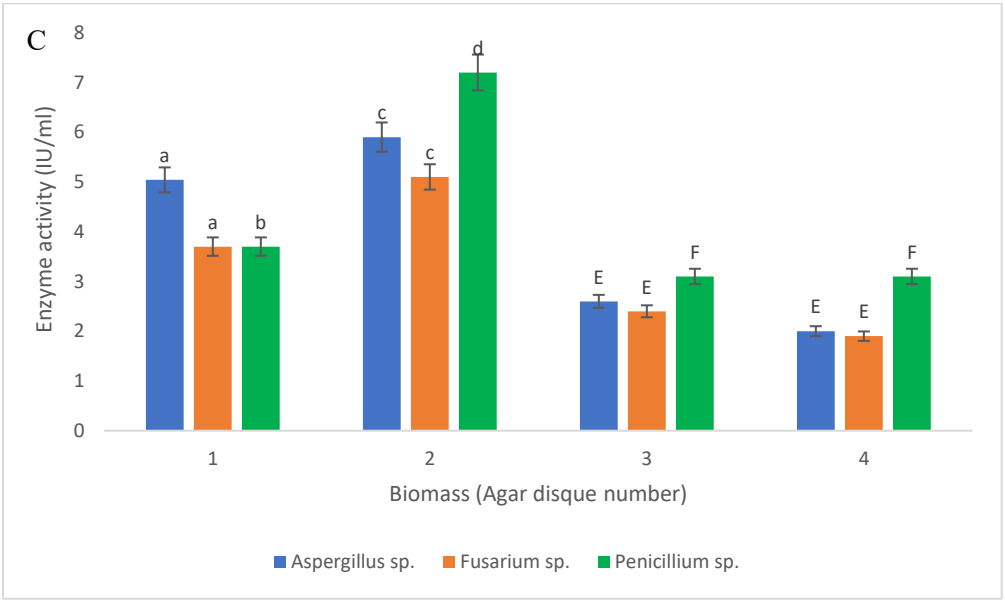
As illustrated in Figure 4D, the optimum temperature for all fungal strains was at 25 °C ± 2. Notably, there was increased activity for *Aspergillus* sp. and *Fusarium* sp. at 45 °C ± 2. *Penicillium* sp. showed no statistically significant difference in activity between 5 °C ± 2, 15 °C ± 2, and 25 °C ± 2.

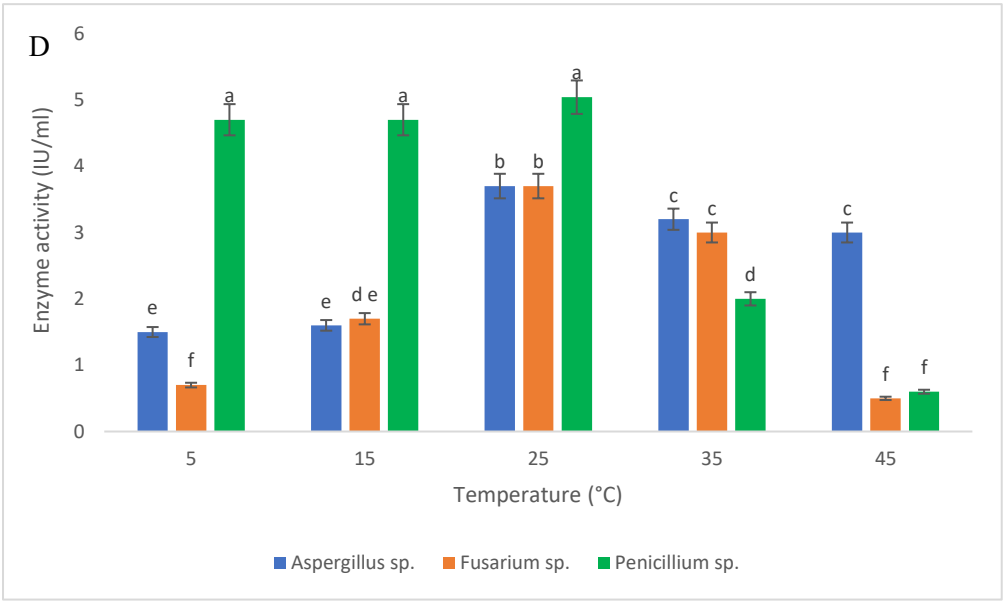
Incubation Period

Lipolytic activity increased for all isolated strains over time,




B
Enzyme activity (IU/ml)
Aspergillus sp.
Fusarium sp.
Penicillium sp.
Control
Strains
with agitation
without agitation


C
Enzyme activity (IU/ml)
Biomass (Agar disque number)
Aspergillus sp.
Fusarium sp.
Penicillium sp.


D
Enzyme activity (IU/ml)
Temperature (°C)
Aspergillus sp.
Fusarium sp.
Penicillium sp.

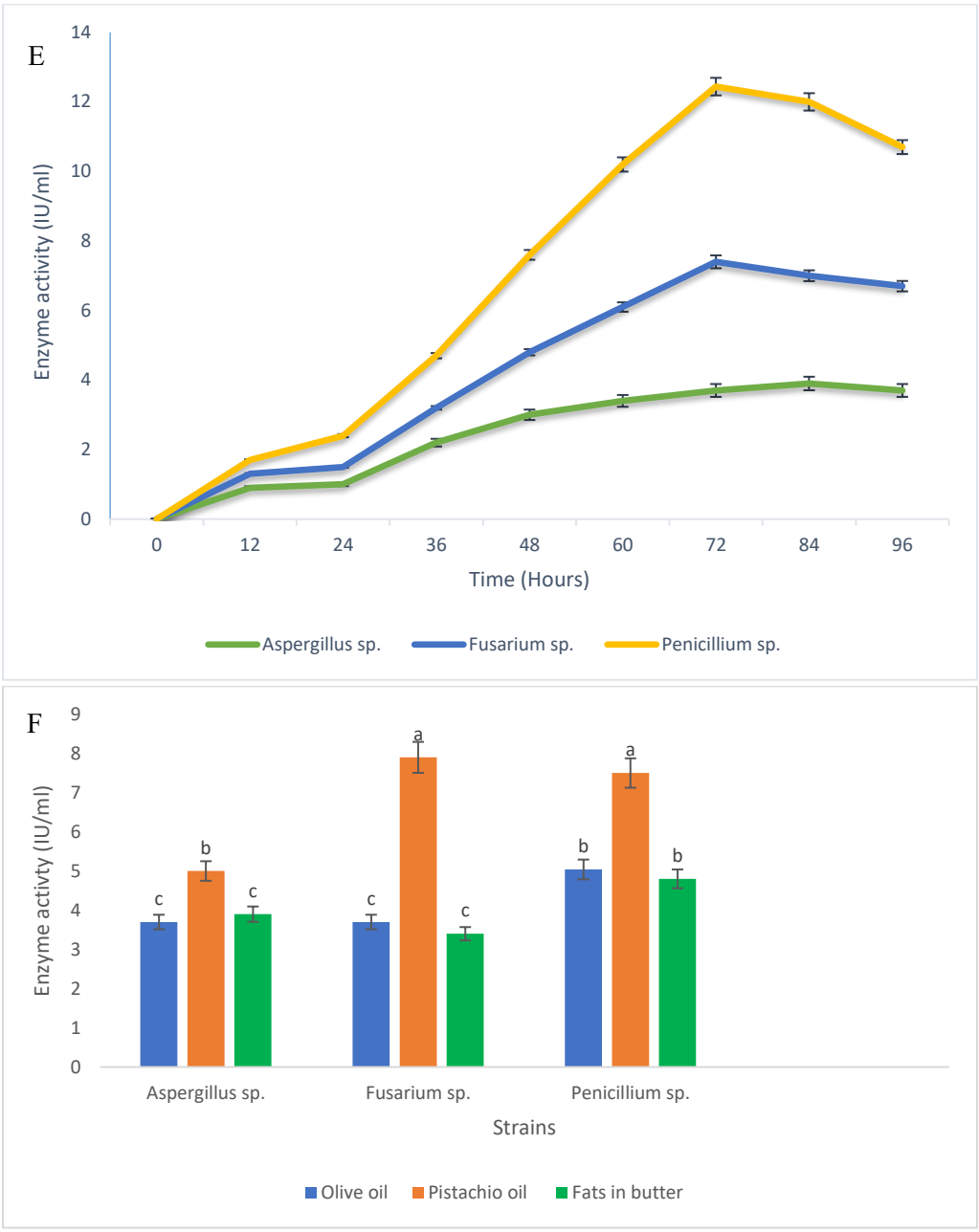


Figure 4. Lipolytic Activity of the Isolated Fungal Strains: **A)** at different pH values; **B)** with and without agitation; **C)** using different concentrations of biomass; **D)** at different temperatures; **E)** during incubation time; **F)** on different fatty substances. Values are mean ± SD. n = 3 in each group. Different letters indicate that samples are significantly different (p<0.05).

starting from 12 hours, reaching a maximum after approximately 72 hours, and then gradually decreasing. *Penicillium* sp. exhibited the highest lipolytic activity during the entire incubation period (Figure 4E).

Lipid Substrates

All tested fungi demonstrated the ability to hydrolyze the three types of fatty substances. *Fusarium* sp. showed the highest activity on pistachio oil (7.9 IU ± 0.15) and *Penicillium* sp. exhibited the highest activity on olive oil (5.4 IU ± 0.43) (Figure 4). Additionally, all three tested fungi showed the ability to hydrolyze the three types of fatty substances. *Fusarium* sp. displayed the highest activity on pistachio oil (7.9 IU ± 0.68), while *Penicillium* sp. showed notable activity on olive oil (5.4 IU ± 0.22) (Figure 4F). The significant hydrolytic activity of pistachio oil did not show any significant difference between the three strains (p<0.05).

Discussion

Many lipases have been isolated from various sources, such as animals, plants and microorganisms, among which microbial lipase is the enzyme with the most diverse enzymatic properties and great industrial application potential. It therefore has promising applications in many

industries, such as food and beverages, waste treatment, biofuels, leather, textiles, detergent formulations and ester synthesis.[24] Lipase enzymes, particularly those derived from microorganisms, are essential biocatalysts with significant industrial importance. Researchers are trying to discover new fungi that produce new enzymes with the ability to degrade more substances in different conditions. Extracellular lipases are mainly exploited extensively because they can be easily separated from the culture media.[25] This study aligned with the goal of isolating and identifying a new source of lipase-producing fungi, potentially contributing to future industrial applications. As previous research has suggested,[26,27] filamentous fungi often showed superior enzyme production under static culture conditions. Identifying the optimal conditions for enzyme activity and stability is crucial for preserving the integrity of the raw enzyme. Each enzyme possesses a specific temperature range at which it functions most effectively. Any deviation from this ideal temperature range can negatively affect enzyme performance. Furthermore, other factors such as pH, substrate concentration, and reaction time play crucial roles in determining the optimal conditions for both enzyme function and stability. These results demonstrate the ability of these enzymes produced by the isolated fungi to maintain their activity at a variety of pHs levels and temperatures, confirming the high stability of these enzymes. This makes them a commercially viable alternative to bacterial enzymes that suffer from a lack of stability.[24] In fact, achieving optimal conditions for enzyme activity involves a delicate balance of factors including temperature, pH, substrate concentration, and reaction time. A careful consideration of these variables is necessary for maximizing the efficiency and stability of the enzyme in various industrial applications. Our results in pH optimisation align with findings from studies conducted by Ramakrishnan et al. (2016) and Bharathi and Rajalakshmi (2019),[28,29] who also observed that an alkaline or slightly neutral pH promotes the production of lipase enzymes. However, it is noteworthy that our study revealed variations, as certain fungi, such as *Penicillium simplicissimum*, demonstrated the ability to produce lipase enzymes not only under alkaline conditions but also in acidic (pH 4.0-6.0) and thermophilic (45-60 °C) environments with high stability. These findings are in agreement with the observations of Greco-Duarte et al. (2023).[30]

Temperature also significantly influences microbial lipase production. Elevated temperatures accelerate chemical reactions and alter the physical properties of cell membranes, thereby influencing the secretion of extracellular enzymes. The choice of an optimum temperature is crucial and plays a vital role in enzyme secretion, especially in the shake-flask method. Previous studies, including those of Mehta et al. (2017) and Bharathi and Rajalakshmi (2019)[6,29] demonstrated that a higher biomass and lipase production were achieved at a temperature of 37 °C. However, it is essential to note that, despite their protein nature, enzymes are sensitive and susceptible to denaturation under extreme temperature conditions.

Thomson et al. (1999)[31] observed that the majority of the isolated moulds exhibited lipolytic activity *in vitro* on a solid oil-based medium supplemented with Tween 80. Additionally, Cesário et al. (2021)[9] conducted a complete factorial experiment, assessing the optimal temperatures for *Penicillium* sp., *Aspergillus* sp., and *A. niger* (28 °C, 32 °C, and 36 °C, respectively).

Results of Rihani et al. (2018)[18] indicated that *Fusarium* sp. exhibited the lowest biomass, which led to better activity, whereas *Aspergillus* sp. demonstrated an average biomass, resulting in good activity. It is noteworthy that an increased spore concentration inoculum not only enhances spore biomass but also leads to a decrease in enzyme production, potentially attributed to limited nutrients in the substrate, as suggested by Iftikhar et al. (2010).[32] Both *Fusarium* sp. and *Aspergillus* sp. demonstrated an average yield of lipases. Consequently, in this study, both strains were categorized as low lipase producers compared to *Penicillium* sp.

Our findings aligned with earlier reports, including those conducted by Kawasaki et al. (1995), Thota et al. (2012), and Rihani et al. (2018).[33,34,18] These studies collectively observed that the maximal secretion of lipase occurred after a 72 hours (3 days) incubation period for two fungal strains, *Penicillium* sp. and *Aspergillus* sp.

The efficacy and activity of the enzyme were investigated in the presence of both powder and liquid commercial detergents at concentrations of 0.1% and 1%. This study is particularly relevant as lipase enzymes employed in detergents must exhibit both activity and stability in alkaline environments (pH 8.0 to 11.0) encountered during rigorous washing conditions.

A prior study conducted by Prazeres et al. (2006)[35] concluded that a lipase produced by *Fusarium oxysporum* enhanced cleaning efficacy when utilized with various commercial detergents. These findings align with the observations made by Barik et al. (2022)[36] who argue that lipases from different fungi exhibited varying behaviours when applied in different biotechnological industries, particularly in processes such as trans-esterification, esterification, and inter-esterification involving various fatty substances. Because of their versatile applications, lipases are commonly used in the detergent industry as additives in washing powders. Additionally, they find utility in the textile industry to enhance fabric absorbency, as well as in the production of biodegradable polymers or compounds. Turati et al. in 2019[37] evaluated the conditions of lipase production from *Penicillium* sp. section *Gracilenta* (CBMAI 1583), concluding that enzyme production was directly related to microbial growth, increasing up to the third day, when it reached the maximum production (0.849 ± 0.15

U/ml and 0.223 ± 0.01 g). Notably, lipase activity is often more pronounced in immobilized enzymes, as reported by Oluwaseun et al. (2023).[38] Additionally, lipases of fungal origin play an interesting role in biotechnology, as many of them are stable over a wide range of pH, at elevated temperatures and in organic solvents.[39]

Researchers have defined various industrial applications of microbial lipases, including the fat and oleochemical industry, detergent industry, production of biodegradable polymers, food processing, flavor development, medical and pharmaceutical industries, pulp and paper industry, biosensors, waste treatment, cosmetics and perfumery and biodiesel production.[40] Fungal lipases are crucial biocatalysts in lipid biotechnology due to their diverse enzymatic properties and substrate specificity, attracting significant research attention.[41]

Conclusion

This study was carried out to isolate and identify fungal strains originating from a novel source to produce lipolytic enzymes. Fungal screening enabled the selection of three strains capable of degrading pistachio oil supplemented into the fermentation medium. While pistachio oil possesses antimicrobial properties, it appears to stimulate lipase production. This soil enriched by this oil may induce the production of specific lipases. Our study successfully identified the most promising fungi for lipolytic activity: *Penicillium* sp., *Aspergillus* sp., and *Fusarium* sp. These strains have the potential to produce lipase under different conditions using lipid substrates.

Authors' Contributions

Conceptualization by ON and BG; Methodology by ON and BY; Software by BS and BY; Validation by BS and BG; Formal analysis by SS and HL; Investigation by BY; Resources by BY; Data curation by ON; Writing—original draft preparation by ON, SS, and HL; Writing—review and editing by ON; Visualization: BS; Supervision and project administration: BG.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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