Archives of Microbiology (2024) 206:264
https://doi.org/10.1007/s00203-024-03997-3

ORIGINAL PAPER

Alkaliphilic and thermostable lipase production by leaf litter fungus *Leptosphaerulina trifolii* A SMR-2011

Mondem Vasundhara[1] · Kirti Singh[1] · Trichur S. Suryanarayanan[2] · Mondem Sudhakara Reddy[1]

Received: 28 November 2023 / Revised: 30 April 2024 / Accepted: 8 May 2024

Abstract
Fungi that inhabit fire-prone forests have to be adapted to harsh conditions and fungi affiliated to Ascomycota recovered from foliar litter samples were used for bioprospecting of molecules such as enzymes. Agni's fungi isolated from leaf litter, whose spores are capable of tolerating 110 ºC were screened for thermostable lipases. One of the isolates, *Leptosphaerulina trifolii* A SMR-2011 exhibited high positive lipase activity than other isolates while screening through agar plate assay using Tween 20 in the medium. Maximum lipase activity (173.2 U/mg) of *L. trifolii* was observed at six days of inoculation and decreased thereafter. Among different oils used, the maximum lipase activity was attained by soybean oil (940.1 U/mg) followed by sunflower oil (917.1 U/mg), and then by mustard oil (884.8 U/mg), showing its specificity towards unsaturated fatty acids. Among the various organic nitrogen sources tested, soybean meal showed maximum lipase activity (985.4 U/mg). The partially purified enzyme was active over a wide range of pH from 8 to 12 with a pH optimum of 11.0 (728.1 U/mg) and a temperature range of 60–80 ºC with an optimal temperature of 70 ºC (779.1 U/mg). The results showed that lipase produced by *L. trifolii* is alkali stable and retained 85% of its activity at pH 11.0. This enzyme also showed high thermal stability retaining more than 50% of activity when incubated at 60 ºC to 90 °C for 2 h. The ions Ca^{2+} and Mn^{2+} induced the lipase activity, while Cu^{2+} and Zn^{2+} ions lowered the activity compared to control. These results suggests that the leaf litter fungus *L. trifolii* serves as a potential source for the production of alkali-tolerant and thermostable lipase.

Keywords Agni's fungi · Alkali tolerant lipase · *Leptosphaerulina trifoli*i · Leaf litter fungi · Thermostable lipase

Introduction

Enzymes are biological catalysts that play an important role by accelerating the chemical reactions. Enzymes do not require expensive heating or cooling systems, and save energy costs, prevent undesired chemical transformation, and loss of volatile compounds; hence they are beneficial in biotechnological applications (Margesin et al. 2003). Enzymes are used in diverse sectors such as food, biofuel, agriculture, pharmaceutical, leather, textile, cosmetics, waste management and so on (Mahfoudhi et al. 2022; Al-Rajhi et al. 2022, 2023). Among these, lipases are extensively used in biotech industries, which include food, production of surfactants, detergents, oil processing, environmental management, pesticides and leather industries (Sharma et al. 2001). Lipases or triacylglycerol ester hydrolases (EC 3.1.1.3) are the carboxylesterases, which catalyze the hydrolysis and synthesis of acylglycerols containing long-chain fatty acids (Jaeger et al. 1999). Lipases show unprecedented levels of activity and are stable both in polar and non-polar environments and perform several reactions such as transesterification, acidolysis, aminolysis, and alcoholysis (Chandra et al. 2020). Lipases are chosen for each application based on their substrates and stability towards temperature and pH. Enzymes with thermal and alkali stability are essential requirements for many industrial applications. Though many lipases have already been described, it is still essential to look for novel lipases with enhanced

Communicated by Nischitha R.

✉ Mondem Sudhakara Reddy
msreddy@thapar.edu

[1] Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab 147004, India

[2] Vivekananda Institute of Tropical Mycology (VINSTROM), Ramakrishna Mission Vidyapith, Chennai, Tamil Nadu 600004, India

Published online: 18 May 2024

substrate specificity, alkali tolerance and thermal stability (Haki and Rahshit 2003).

Lipases produced by microbes have gained more attention from the industry due to their flexibility in pH, temperature, and organic solvent (Verma et al. 2012; Chandra et al. 2020). Most of the lipases applied in biotech industries are derived from bacteria and fungi. Fungal lipases have gained more attention in the industries because of their extracellular secretion, substrate specificity and stability under varied chemical and physical conditions and are more adapted to less moisture content than bacteria and yeast in solid-state fermentation (Hölker et al. 2004). Fungal enzymes are extracellular in nature and they can be extracted easily, which significantly reduces the cost and makes this source preferable over bacteria (Mehta et al. 2017). Different fungi, such as *Aspergillus niger, Candida rugosa, Humicola lanuginosa, Mucor miehei, Rhizopus* spp., are capable of producing lipases in large scale and has given a favorable vision in meeting the needs of many bio-industries (Chandra et al. 2020). These lipases shed light on various biotechnological fields such as agrochemical, food, dairy products, pharmaceutical, biodiesel production, paper and leather, detergent industries and in bioremediation along with their use in some medical applications.

The enzymes used in industries should be robust and tolerant to extreme conditions such as high temperature, pH, osmolarity, pressure, etc. Thermophilic fungi are potential sources of enzymes with thermostability and activity levels (Yadav et al. 2022; Abdel-Ghany et al. 2019). There is dearth of information on lipases from this source as compared to other thermophilic organisms such as eubacteria and archaea (Diaz et al. 2006). Fungi isolated from extreme environments are capable of producing enzymes tolerant to these harsh conditions (Bhadra et al. 2022). Previously, we have isolated some of the leaf litter fungi from litter samples (Agni fungi), whose spores are able to survive after exposure to 100 °C for 2 h (Suryanarayanan et al. 2011). These organism might serve as potential sources for the production of some industrially important enzymes such as lipases, which can resist extreme industrial conditions like high temperature and pH.

The present study was aimed to inestigate lipase production by *Leptosphaerulina trifolii* A SMR-2011 which is a fungus that was previously isolated from leaf litter. The lipase enzyme produced by this fungus was characterized for its tolerance to different pH and temperatures.

Materials and methods

Isolation of leaf litter fungi

The foliar samples of litter of different hosts were collected from the private land of dry deciduous forest of Nilgiri Biosphere Reserves, Western Ghats (10°50′ N and 12°16′ N latitude and 76°00′ E to 77°15′ E longitude) and used for the isolation of fungi. About 50% of the Nilgiri Biosphere forest areas are prone to forest fires (Vijayan et al. 2020). Twenty-five leaf litter fungi were isolated mostly in their anamorphic states, and induced sporulation by cultivating them on potato dextrose agar (PDA) at 28 °C for 7 d under a 9 h: 15 h of light and dark cycle. The spores obtained from these fungi were exposed to 100 °C in a preheated hot air oven to test their survivability and found that the spores of nine fungi were regenerated after 2 h of exposure at 100 °C (Suryanarayanan et al. 2011. The microscopic characterization of these fungi were described in our previous study (Suryanarayanan et al. 2011).

Screening for lipase production

All nine isolates of leaf litter fungi, whose spores survived high temperatures were screened for lipase production according to the method described in Sierra (1957). The fungi were grown on a medium consisting of peptone 1% (w/v), NaCl 0.5% (w/v), $CaCl_2$ 0.1% (w/v), bacteriological agar 1.5% (w/v), and Tween 20 1.0% (v/v) in Petri plates at 28 °C for 7 days. Precipitation around the fungal colony indicated the production of lipase (Rai et al. 2014). This method is based on the principle of calcium salt precipitation. The hydrolysis of tween releases fatty acids, which bind with the calcium in the medium to form insoluble crystals around the fungal colony. The isolate showing high positive activity was further selected for molecular identification, lipase production and characterization (Fig. 1S).

Identification of leaf litter fungi

The identification of these fungi were confirmed by internal transcribed spacer (ITS) region of rRNA. Genomic DNA was isolated as per the method described in Van Kan et al. (1991) and ITS region was amplified using ITS1 (5'-TCC GTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGC TTATTGATATGC-3') primers. The amplified ITS products were sequenced and the sequence analysis was performed to identify the homologous sequences in NCBI database using BLASTN program. Phylogenetic tree was reconstructed by neighbour-joining (NJ) method using MEGA 11 software (Tamura et al. 2021). The fungal isolates were deposited at the National Fungal Culture Collection of India (NFCCI),

Agharkar Research Institute, Pune, India and the ITS sequences were deposited in NCBI. The accession numbers of the cultures as well as the GenBank accession numbers of ITS sequences are given in Table 1.

Lipase production by submerged fermentation

Three mycelial discs (5 mm diam) cut from the margin of actively growing colony of *Leptosphaerulina trifolii* A SMR-2011 was inoculated into a basal medium (peptone 0.5 g; $MgSO_4.7H_2O$ 0.05 g; KCl 0.05 g; KH_2PO_4 0.2 g; $NaNO_3$ 0.05 g; Olive oil 1.0 ml; and distilled water 1000 ml; pH 6.5) in 250 ml Erlenmeyer flasks, and incubated at 28 ºC in a rotatory shaker at 120 rpm. After six days, the contents were centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was used (crude extract) to assess lipase activity by p-nitrophenyl palmitate (p-NPP) method as described in Licia et al. (2006). Briefly, the substrate was prepared by adding 1 ml of solution A (40 mg of p-NPP dissolved in 12 ml isopropanol) dropwise to 19 ml of solution B (0.1 g of gum arabic and 0.4 ml of Triton X-100 dissolved in 90 ml of distilled water) with constant stirring to obtain an emulsion. The assay mixture consisted of 1.0 ml of the substrate, 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.0), 0.1 ml of the crude extract, and the final volume was made to 4 ml with distilled water. The contents were incubated at 40 ºC for 30 min, and 0.2 ml of isopropanol was added to stop the enzyme activity, and the absorbance was recorded at 410 nm. The p-nitrophenol prepared in 0.1 M potassium phosphate buffer (pH 7.0) was used to prepare the standard graph. The maximum activity of enzyme data was assumed to be 100%, and relative enzyme activity was determined for each data. The specific enzyme activity of the enzyme (U/mg protein) was calculated by dividing the enzyme activity value by protein content (mg/ml). Bradford assay was performed to determine the protein content (Bradford 1976). The amount of enzyme that released 1.0 µM p-NP per minute under the assay conditions is defined as one unit (U) of the enzyme (Maia et al. 1999).

Enzyme optimization and characterization

Optimal time for lipase production

L. trifolii was grown in a basal medium along with 1% olive oil (v/v) as an enzyme substrate and carbon source by incubating at 28 °C in a rotatory shaker at 120 rpm. Aliquots of the culture broth were withdrawn every two-day intervals up to 10 days. The contents were centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was used to determine lipase activity. The maximum lipase activity was recorded on the 6th day, and hence, for further experiments, a 6-day-old fungal culture broth was used.

Effect of glucose on lipase production

Glucose is known to inhibit the hydrolysis activities of many lipases (Tsuzuki et al. 1999). To test whether glucose have any impact on the lipase production by *L. trifolii*, the fungus was grown in presence of glucose, olive oil and glucose olive oil combination. The fungus was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of basal medium supplemented with 4 ml of 20% glucose solution (w/v), 1% olive oil (v/v) separately and in combination. The fungus was grown for six days at 28 °C in a rotatory shaker at 120 rpm. After six days, the fungal culture was centrifuged, and assessed the lipase activity from the supernatant sample.

Effect of different oils and organic nitrogen sources on lipase production

The influence of different vegetable oils were tested on lipase production using them as carbon sources and inducers. *L. trifolii* was grown in a basal medium supplemented with different vegetable oils like sunflower oil, soybean oil, mustard oil, and coconut oil for six days of fermentation. These were added separately to the basal medium in place of olive oil (1% v/v). Organic nitrogen sources are generally used to produce higher yields of lipase by microorganisms than inorganic sources. Hence, different organic nitrogen sources such as urea, casein, yeast extract, and soybean meal were added in place of peptone (0.5% w/v) in the basal medium. The fungus was grown for six days at 28 °C in a rotatory shaker at 120 rpm. After six days, the fungal culture was centrifuged and the supernatant was used to assess the lipase activity (Licia et al. 2006).

Partial purification of lipase

Partial purification of lipase was carried out according to the method described in El-Ghonemy et al. (2017). The crude enzyme was extracted as mentioned above and precipitated by adding various mass fractions of ammonium sulfate solution by bringing to 70 to 80% saturation with constant stirring at 4 ºC at 24 h. The precipitated proteins were separated by centrifugation at 10,000 g for 15 min at 4 °C, suspended in a minimal volume of (5 ml) of 0.1 M Tric HCl buffer (pH 8.0) and dialized against the same buffer by placing it in a cellulose membrane bag for 24 h at 4 ºC. The precipitate was collected after dialysis and centrifuged 10,000 g for 10 min at 4ºC. About 40–60% of fractions, with maximum lipase activity, were collected and used for protein

content and lipase activity. Protein content in the fractions was determined by Bradford assay (Bradford 1976) and the lipase activity by according to the method described in Licia et al. (2006).

Enzyme characterization

Effect of pH on lipase activity and stability

To investigate the alkaliphilic nature of lipase, the partially purified enzyme was incubated at a varied range of pH from 4.0 to 12.0 using different buffers (100 mM). For pH 4.0 to 5.0, sodium acetate buffer, for pH 6–7, potassium phosphate buffer, for pH 8.0, Tris-HCl buffer and for pH 9–12, glycine-NaOH buffer were used. The reaction mixture (500 µl each buffer, 1 ml substrate, and 100 µl partially purified enzyme) was incubated at 40 °C for 30 min and assessed the lipase activity. Stability assay was carried out by incubating the partially purified enzyme at 40 °C for 2 h in different buffers (100 mM). The residual activity was then determined by p-NPP method (Licia et al. 2006).

Effect of temperature on lipase activity and stability

To determine the optimum temperature for lipase, the partially purified enzyme was incubated at different temperatures (30 °C to 100 °C) for 30 min in standard reaction mixure. The lipase activity was assessed by p-NPP method (Licia et al. 2006). The thermal stability of the enzyme was evaluated by incubating partially purified enzyme at different temperatures (30 °C to 100 °C) for 2 h. The residual activity of lipase was then determined.

Effect of metal ions on lipase activity

The influence of metal cations and the chelating agent ethylenediaminetetraacetic acid (EDTA) on the partially purified lipase activity was evaluated. The enzyme was incubated for 30 min at 70 °C with Ca^{2+}, Mn^{2+} as $CaCl_2$ and $MnCl_2$ salts, Cu^{2+}, Zn^{2+} as $CuSO_4$ and $ZnSO_4$ salts, and EDTA, all at 1.0 mM concentration. Lipase activity was determined with and without the addition of metal salts or EDTA.

Statistical analysis

Three replicates were used for all the experiments. Analysis of variance was used to analyze the data and the significant differences among the mean values were determined by Tukey's test at $P < 0.05$. Graph Pad Prism 8.1 software was used to analyze all the data.

Results

Isolation and identification

Twenty five isolates of leaf litter fungi were isolated from the foliar litter samples collected from different host plants. The majority of the fungi isolated were belong to Ascomycota and were in their anamorphic states. Among these , the spores of 9 leaf litter fungi exposed to 100 °C for 2 h survived when regenerated on PDA medium. These leaf litter fungi were identified by their microscopic and ITS of rRNA sequence analysis. Sequence analysis using BLASTN led to the identification of these fungal isolates as *Bartalinia pondoensis*, *Chaetomella raphigera*, *Curvularia* sp., *Exserohilum rostratum*, *Leptosphaerulina trifolii*, *Pithomyces* sp., *Pestalotiopsis microspora*, and *Phoma* sp. Phylogenetic analysis confirmed the identity by clustering with their homologus groups (Fig. 1). The accession numbers of the cultures deposited at the National Fungal Culture Collection of India (NFCCI) and GenBank accession numbers of ITS sequences are given in Table 1.

Lipase production

Among nine leaf litter fungi, *Leptosphaerulina trifolii* exhibited maximum lipase activity as determined by plate assay (Table 1). The other isolates such as *B. pondoensis*, *C. raphigera*, *Montagnulaceae* sp., *P. microspora* and *Phoma* sp. showed moderate activity, while the other isolates, *P. microspora* and *Curvularia* sp. did not exhibit the lipase activity (Table 1). *Leptosphaerulina trifolii* was chosen for further studies based on its high lipase activity and faster growth.

Optimal time for lipase production

To find the optimal time for higher lipase production, *L. trifolii* was grown at different time periods. The lipase activity increased up to 6th day and decreased thereafter (107.2, 153.2, 173.2, 153.9 and 128.7 U/mg for 2, 4, 6, 8 and 10 days, respectively) with maximum activity being on the 6th day (Fig. 2). The pH of the culture filtrate did not change significantly, where it reduced from 6.9 to 6.7 at the end of the 10th day (Fig. 2).

Effect of glucose on lipase production

To find the effect of glucose on lipase activity, *L. trifolii* was grown in the presence and absence of glucose along with 1% olive oil. The results showed that basal media supplemented with olive oil alone significantly increased the lipase activity compared to glucose and glucose olive

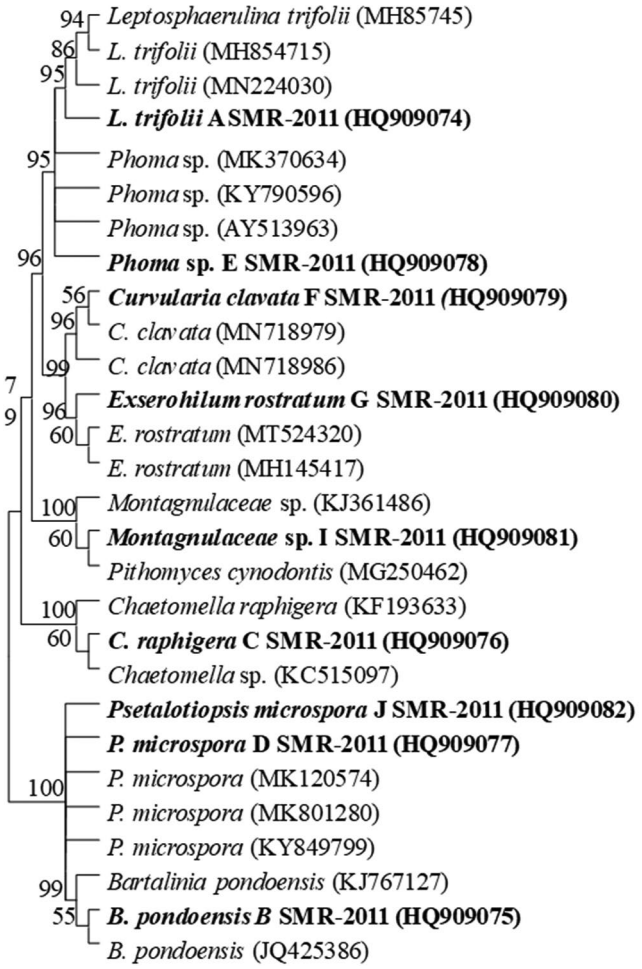

Fig. 1 Phylogenetic tree reconstructed by neighbour-joining method showing the relationship between ITS sequences of leaf litter fungi of the present study and those of related sequences from Genbank of NCBI. Accession numbers are mentioned in parentheses. Numbers at the nodes are bootstrap values

oil combination. The lipase activities observed for olive oil alone, glucose alone and a combination of glucose and olive oil are 181.9 ± 8.2 U/mg, 156.8 ± 2.8 U/mg, and 175.6 ± 4.6 U/mg, respectively.

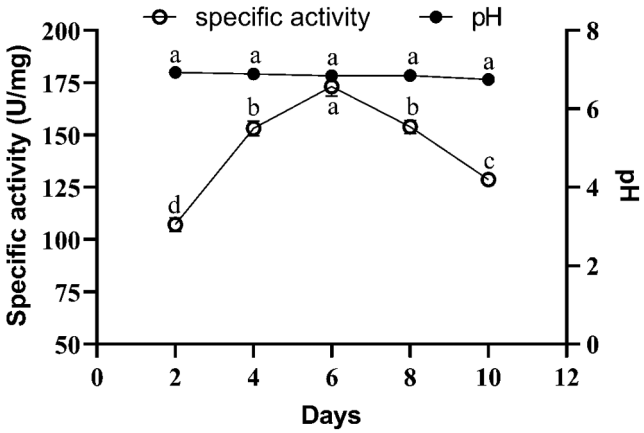

Fig. 2 Effect of incubation time on lipase production and pH change of *Leptosphaerulina trifolii* grown under submerged conditions. Values sharing different letters within the treatment are significantly different at $p < 0.05$

Effect of different oils and organic nitrogen sources on lipase production

Lipase activity was investigated by growing the fungus supplemented with different oils. The lipase showed greater activity for oils with a higher percentage of unsaturated fatty acids. The highest lipase level was observed with 1% soybean oil (940.1 U/mg) followed by sunflower (917.1 U/mg) and mustard oil (884.8 U/mg) compared to basal medium (785.3 U/mg). Coconut oil showed the least lipase activity of 687.1 U/mg (Fig. 3a). When tested with different organic nitrogen sources for maximum lipase production, soybean meal showed maximum lipase activity (985.4 U/mg), followed by yeast extract (878.9 U/mg). Casine hydrolysate and urea showed less lipase activity than the basal medium (Fig. 3b).

Table 1 Different fungi isolated from the leaf litter samples, their hosts, culture accession numbers, GenBank accession numbers of ITS sequences and production of lipase activity by plate assay

Fungal isolates	Host	GenBank accession no.	Culture accession no.	Lipase activity
Bartalinia pondoensis B SMR-2011	*Bridelia retusa*	HQ909075	NFCCI2307	+
Chaetomella raphigera C SMR-2011	*Pterocarpus marsupium*	HQ909076	NFCCI2308	+
Curvularia sp. F SMR-2011	*Holoptelia integrifolia*	HQ909079	NFCCI2311	-
Exserohilum rostratum G SMR-2011	*Bridelia retusa*	HQ909080	NFCCI2312	-
Leptosphaerulina trifolii A SMR-2011	*Holoptelea integrifolia*	HQ909074	NFCCI4854	++
Montagnulaceae sp. I SMR-2011	*Sapindus emarginatus*	HQ909081	NFCCI4855	+
Pestalotiopsis microspora D SMR-2011	*Maytenus emarginatus*	HQ909077	NFCCI2309	-
P. microspora J SMR-2011	*Syzygium cumini*	HQ909082	NFCCI2313	+
Phoma sp. E SMR-2011	*Butea monosperma*	HQ909078	NFCCI2310	+

++: High activity; +: Moderate activity; -: No activity

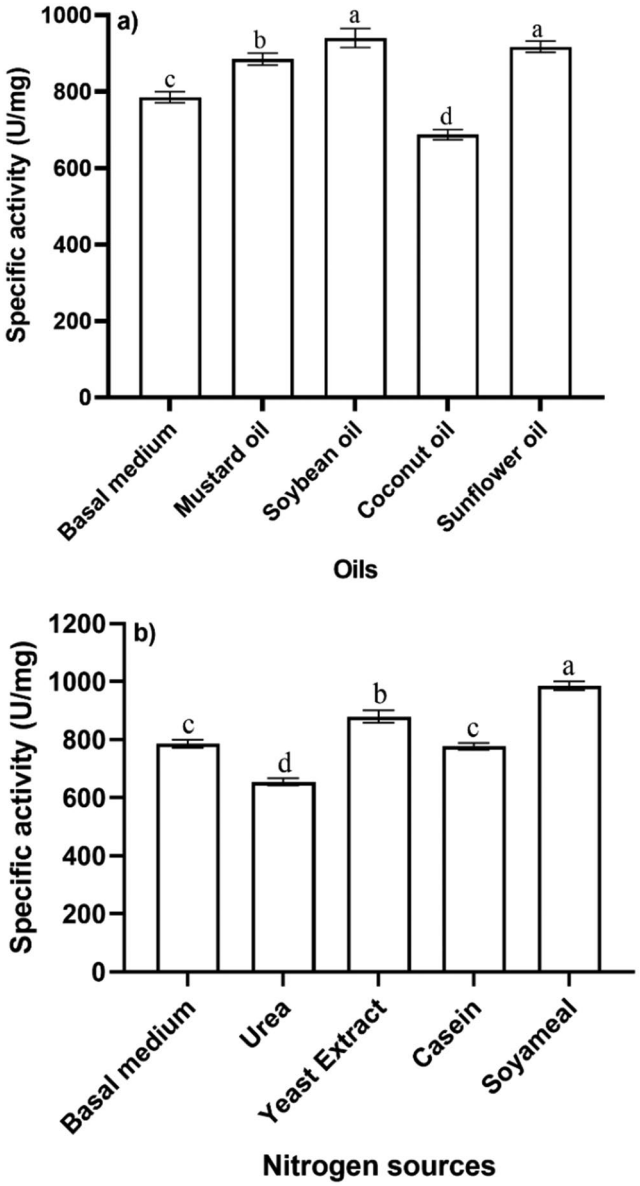

Fig. 3 Influence of (**a**) different oils and (**b**) organic nitrogen sources on the lipase activity of *Leptosphaerulina trifolii* grown in submerged fermentation. Bars sharing different letters are significantly different at $p < 0.05$

Partial purification and characterization of lipase

Results presented in Table 2 clearly demonstrated that the purification of lipase enzyme from *L. trifolii* through ammonium sulphate precipitation and dialysis showed a 4.5 fold purification with 42.1% recovery. Besides, the total protein mass decreased from 355 mg/ml to 33.2 mg/ml, while the specific activity increased from 173.2 U/mg to 779.9 U/mg.

Effect of pH and temperature on lipase production

The partially purified lipase activity by *L. trifolii* was active over a broad pH range of 8.0 to 12.0, and lower at pH 4.0 to 7.0. The partially purified lipase showed its optimum activity at pH 11.0. The lipase activity of pH 11.0 was considered as 100% relative activity with specific activity of 728.1 U/mg (Fig. 4a). The lipase activity was significantly reduced when the pH levels were below 6.0. The pH stability was estimated by incubating the partially purified enzyme for 2 h at 40 °C. These results indicated that lipase is an alkaline pH stability enzyme which was more stable at pH 8.0 – 12.0 and the maximum stability was recorded at pH 11.0, where it retained its 85% activity (Fig. 4a). The optimal temperature of partially purified lipase was determined by assessing the enzyme activity at varied temperatures (30, 40, 50, 60, 70, 80, 90 and 100 °C) at pH 11.0. The enzyme exhibited its optimal activity at 70 °C and the lowest at 30 °C. Lipase activity at 70 °C was considered as 100% relative activity, which showed a specific activity of 779.1 U/mg). Partially purified lipase was more stable between 60 and 90 °C and more than 50% of its activity was retained in these temperatures after 2 h of incubation. At 70 °C, the partially purified enzyme retained 72% of activity after 2 h of incubation (Fig. 4b).

Effect of metal ions on lipase activity

Cations such as Ca^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} and EDTA on the lipase activity of partially purified lipase were examined. The results showed that Ca^{2+} and Mn^{2+} ions induced the lipase activity, while Cu^{2+} and Zn^{2+} lowered compared to the control. The maximum activity of lipase was recorded in the presence of Mn^{2+} ions (243.2 U/mg) compared to other

Table 2 Yield and fold of purification of lipase produced by *L. trifolii*

Sample	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Yield of activity (5)	Fold of purification
Crude extract	355.2	61,486	173.2	100	1
Ammonium sulphate fraction 40–80%	33.2	25,892	779.9	42.1	4.5

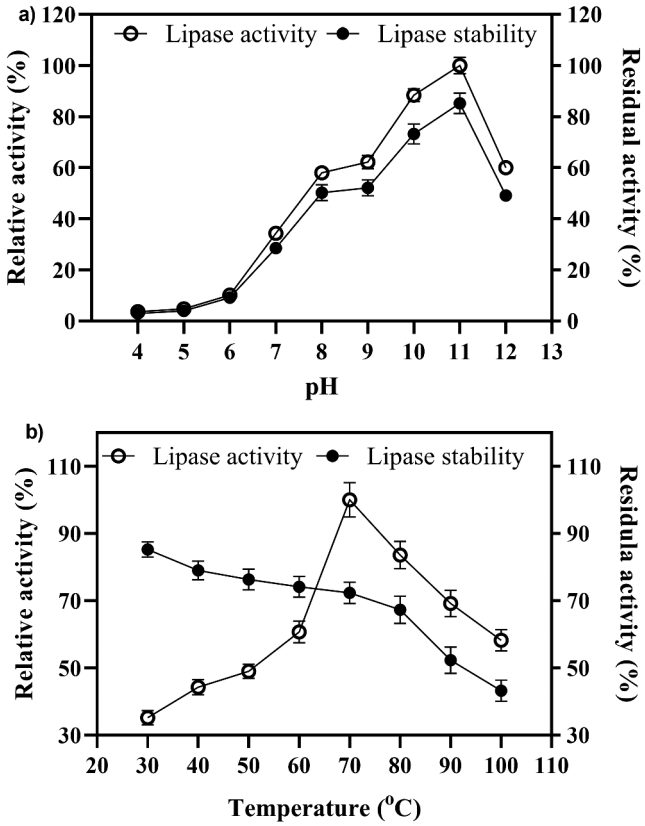

Fig. 4 Characterization of partially purified lipase activity from *Leptosphaerulina trifolii* (**a**) Lipase activity and stability at different pH. The data are shown as relative values taking maximum activity obtained at pH 11.0 and 40 °C (728.1 U/mg), and (**b**)) Lipase activity and stability at different temperatures The data are shown as relative values taking maximum activity obtained at 70 oC and pH 11.0 (779.1 U/mg)

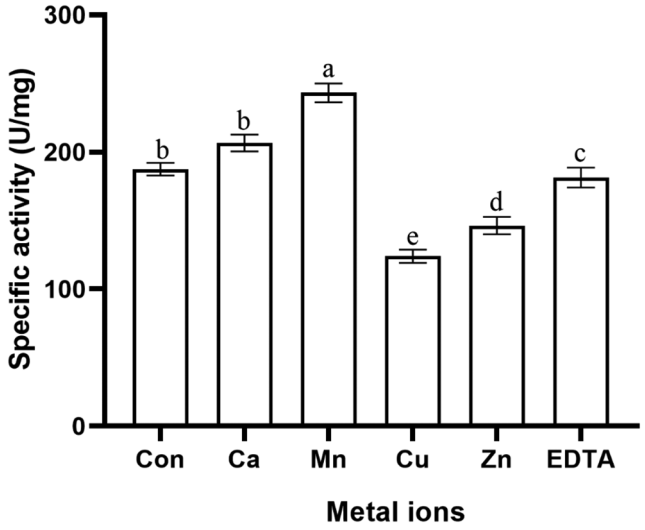

Fig. 5 Influence of different metal ions and EDTA on partially purified lipase activity of *Leptosphaerulina trifolii*. Bars sharing different letters are significantly different at $p < 0.05$

ions. The chelating agent EDTA did not affect the lipase activity compared to control (Fig. 5).

Discussion

Fungi are capable of producing several enzymes for their survival within a wide range of substrates.

The production of fungal lipases are affecteded by nutritional and physico chemical factors, presence of lipids, inorganic salts and more importantly the fungal strains (Mehta et al. 2017). Use of fungal lipases as biocatalysts for the production of various biomolecules has a tremendous potential benefit for future developments. The major genera of filamentous fungi which produce lipases are *Rhizopus, Aspergillus, Penicillium, Mucor, Geotrichum, Humicola, Rhizomucor, Fusarium, Acremonium*, and *Alternaria* (Mehta et al. 2017). In the present study, *Leptosphaerulina trifolii* A SMR-2011 was selected for the lipase production because of its thermotolerance and also explore the leaf litter fungi for the enzyme production.

Lipase production is mainly affected by carbon sources as they have been reported as suitable inducers for extracellular lipases (Fickers et al. 2004). Various vegetable oils have been reported to serve as carbon sources as well as inducers for lipase production (Mehta et al. 2017). Fatty acids of vegetable oils are metabolized by lipases through β-oxidation by hydrolyzing vegetable oil triacylglycerides in the medium (Obradors et al. 1993). Salihu et al. (2011) reported the highest lipase activity by *Candida cylindracea* in presence of olive oil (4.02 U/ml) compared to corn (3.31 U/ml), sunflower (2.99 U/ml) and palm oil (2.78 U/ml). The highest hydrolytic activity (28 U/ml) of lipase produced by *Preussia africana* was observed with sunflower oil compared to cotton, palm kernel, corn, canola and linseed oil (Oliveira et al. 2021). Rajendran et al. (2008) reported that olive oil had significant influence on lipase production (3.8 U/ml) in *Candida rugosa*. *Aspergillus* sp. F18 isolated from the oily residues showed maximum yield of lipase (110 mg/ml) in presence of 2% soybean oil. Canseco-Pérez et al. (2018) isolated *Trichoderma harzianum* from plant debris of banana plantation, which exhibited lipolytic activity of 205 U/ml in presence of olive oil. In the present study *L. trifolii* exhibited maximum lipase activity in presence of soybean oil 940 (U/mg), followed by sunflower oil (917.1 U/mg). The lipase produced by *L. trifolii* showed higher specificity for unsaturated fatty acids present in soybean, sunflower and mustard oil than saturated fatty acids present in coconut oil. Similar results were reported by Oliveira et al. (2021), where lipase exhibited greater activity for oils that had higher percentage of unsaturated fatty acids in their composition. The substrate specificity of lipase produced by

L. trifolii might be related to the substrate being used as an inducer, which explains the soybean oil, sunflower and mustard oil have more than 85% unsaturated fatty acids compared to coconut oil with 90% saturated fatty acids. Stránský et al. (2007) reported that due to substrate specificity, lipase can recognize and hydrolyze rapidly the certain esters present in fatty acids, which are bound to triacylglycerides.Various organic nitrogen sources influence the cell growth as well as lipase production. Fickers et al. (2004) reported the maximum lipolytic production by the yeast *Yarrowia lipolytica* in presence of tryptone N1 (484.7 U/mg) followed by petone E1 (109 U/mg) compared to different other nitrogen sources. *Fusarium solani* F61 grown in culture medium supplemented with tryptone increased lipase production by 461.3% and by 419.4% with yeast extract (Mendes et al. 2019). Al-Meshal and Suliman (2022) reported that the lipase production by *Aspergillus niger* increased from 22.86 U/ml to 36.93 U/ml due to the addition of peptone to the mdedium. Panuthai et al. (2012) reported maximum lipase production by *Fusarium oxysporum* in presence of peptone (2.2 U/ml) and soybean powder (2.1 U/ml). In the present study, *L. trifolii* exhibited maximum lipase production in presence of soybean meal (985.4 U/mg) followed by yeast extract (878.9 U/mg) among the nitrogen sources tested, which were significantly higher than the basal medium alone (785.7 U/mg). These results suggests that both carbon and nitrogen sources play an important role in lipase production.

Many studies have reported that fungal lipases are active and stable at temperatures ranging from 30 °C to 40 °C (Ali et al. 2023). Some other studies indicated that the lipases extracted from thermotolerant fungal isolates exhibited their activities at higher temperatures. Alabdalall et al. (2021) reported the optimi\um lipases activity for two fungal isolates *A. niger* MH078571.1 and *A. niger* MH079049.1 were 45ºC (794.2 U/ml) and 55 ºC (796.9 U/ml), respectively. The lipase produced by thermophilic fungus *Thermomyces lanuginosus* showed its optimium activity at 40 ºC with 100% relative activity (Qu et al. 2022). Ezenwelu et al. (2022) reported that the optimal temperature of lipase produced by *Aspergillus* sp. was 70 µmoles/min at 60 ºC compared to other temperatures. Facin et al. (2018) reported that the relative activity of lipase isolated from thermotolerant and organic solvent resistant *T. lanuginosus* showed its optimal activity at 60 °C and retained 90% of its activity at 60 °C for 24 h. In the present study, lipase produced by a thermotolerant *L. trifolii*, showed its optimal relative activity at 70 ºC (779.1 U/mg) and retained more than 50% of its activity till 90 ºC for 2 h. Previous studies have revealed that fungal lipases in general are active and stable in a wide range of pH (Kumar et al. 2023). The extracellular lipase produced by *Peziza* sp. showed its maximal activity at pH 8.0 (4.92 U/ml) and reduced at pH 6.0, 10 and 11.0 and showed its stability from pH 8.0 to10.0 (Ktata et al. 2020). The lipase produced by *Trichosporon* sp. showed its optimal relative activity at pH 8.0 and stability at pH 7.0 to 9.0 (Cao et al. 2020). Lipase produced by *A. niger* MH078571.1 and *A. niger* MH079049.1 showed its optimal activity (795.39 and 795.77 U/ml, respectively) and stability at pH 8.0 (Alabdalall et al. 2021. Qu et al. (2022) reported the optimal relative lipase activity and stability at pH 9.0 for the lipase produced by thermophilic fungus *Thermomyces lanuginosus*. The lipase produced by *L. trifolii* in this study also showed its maximum activity at pH 11.0 (728.1 U/mg) pH stability at pH 8.0 to 12.0, indicating this enzyme as pH stable enzyme retaining 85% activity at pH 11.0.

Metal ions play various important roles by influencing the structure and function of enzymes including lipases (Mahfoudhi et al. 2022). Ezenwelu et al. (2022) reported increased lipase activity of *Aspergillus* sp. in presence of Ca^{2+} (68 µmoles/min) and Co^{2+} (69 µmoles/min) compared to control (62 µmoles/min). A lipase purified from *Aspergillus carbonarius* NRRL 369 increased its activity by 240% in presence of Mn^{2+}, 150% in presence of Ca^{2} and Zn^{2+}, and 180% with Co^{2+} compared to control (Panojotova et al. 2023). Hafez et al. (2024) reported that metal ions such as Cu^{2+} increased the lipase activity (9.14 U/mg), while Zn^{2+} (4.95 U/mg), and Mn^{2+} (5.22 U/mg) lowered the activity compared to control (8.54 U/mg). In the present study, Mn^{2+} (243.3 U/mg) and Ca^{2+} 206. 7 U/mg) increased the lipase activity, while Zn^{2+} (146.4 U/mg), Cu^{2+} (123.9 U/mg) and EDTA (181.4 U/mg) lowered compared to control (187.5 U/mg). These results suggests various metal ions affect the lipases activity and is influenced by the type of metal ions, and the type of fungal strains.

Conclusions

The leaf litter fungi isolated from forest fire-prone areas capable of tolerating high temepratures were exploredfor the first time for the production of lipases. One of the isolates, *L. trifolii* is capable of producing lipase under submerged conditions with maximum enzyme activity after six days of cultivation in a basal medium using olive oil as a carbon source. Soybean oil supported the maximum lipase production (940.1 U/mg) followed by sunflower oil (917.1 U/mg) showing its specificity towards unsaturated fatty acids. The enzyme was active over a broad range of pH 8.0 to 12.0, with maximum lipase activity at a pH of 11.0. These results suggest that lipase produced by *L. trifolii* is an alkaline pH-stable enzyme where it retained 85% of its activity at pH 11.0. The lipase exhibited its optimal activity at 70 °C and was stable between 60 ºC and 90 °C, retaining

more than 50% of activity in 2 h of incubation. The lipase production was increased in the presence of Mn^{2+} and Ca^{2+} ions. These results suggests that lipase produced by *L. trifolii* have the alkaline stability and thermostability compared to many lipaases produced by various fungi. Also, leaf litter fungi isolated from fire-prone forest areas serves as a potential source for thermostable and alkali-tolerant enzymes. Further, investigations are needed to purify the enzyme and optimize the conditions for maximum lipase production.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-024-03997-3.

Acknowledgements The authors are thankful to Thapar Institute of Engineering & Technology, Patiala and Swami Shukadevananda, Secretary, Ramakrishna Mission Vidyapith, Chennai, for the facilities provided.

Author contributions MS Reddy and TS Suryanarayanan designed the study; Kirti Singh and M Vasundhara conducted the experiments. Data analysis was done by M Vasundhara and MS Reddy. The first draft of the manuscript was prepared by M Vasundhara and TS Suryanarayanan and all authors commented on previous versions of the manuscript. All authors read and approved the final version of the manuscript.

Funding This work did not receive any funding from external sources.

Data availability The authors declare that all data supporting the findings of this study are available within this article and the ITS sequence information at the NCBI database.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to Publication Not applicable.

Competing interests The authors declare no competing interests.

References

Abdel-Ghany TM, Alawlaqi MM, Shater AM, Mohamed A, Al-Abboud MA (2019) Congo red biosorption with live and dead biomass of thermophilic *aspergillus fumigatus*. Egypt J Exp Biol (Bot) 15:1–6. https://doi.org/10.5455/egyjebb.20181206084342

Al-Meshal A, Suliman A (2022) The Effect of some components changes of *aspergillus Niger* culture media on the production of lipase enzyme. J Biobased Mater Bioenergy 16:402–407. https://doi.org/10.1166/jbmb.2022.2198

Al-Rajhi AMH, Alharbi AA, Yahya R, Abdel Ghany TM (2022) Induction of hydrolytic enzyme production and antibiosis via a culture of dual fungal species isolated from soil rich with the residues of woody plants in Saudi Arabia. BioResources 17:2358–2371. https://doi.org/10.15376/biores.17.2.2358-2371

Al-Rajhi AMH, Alawlaqi MM, Abdel Ghany TM, Moawad H (2023) *Amanita* sp. from subtropical region of Saudi Arabia as a source of chitinase enzyme and its antifungal activity. BioResources 18:2928–2939. https://doi.org/10.15376/biores.18.2.2928-2939

Alabdalall AH, Al-Anazi NA, Aldakheel LA, Amer FH, Aldakheel FA, Ababutain IM, Alghamdi AI, Al-Khaldi EM (2021) Application and characterization of crude fungal lipases used to degrade fat and oil wastes. Sci Rep 11:19670. https://doi.org/10.1038/s41598-021-98927-4

Ali U, Anwar Z, Hasan S, Zafar M, Ain Nu, Afzal F, Khalid W, Rahim MA, Mrabti HN, AL-Farga A, Eljeam HARA (2023) Bioprocessing and screening of indigenous wastes for hyper production of fungal lipase. Catalysts 13:853. https://doi.org/10.3390/catal13050853

Bhadra F, Gupta A, Vasundhara M, Reddy MS (2022) Endophytic fungi: a potential source of industrial enzyme producers. 3 Biotech 12:86. https://doi.org/10.1007/s13205-022-03145-y

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1006/abio.1976.9999

Canseco-Pérez MA, Castillo-Avila GM, Chi-Manzanero B, Islas-Flores I, Apolinar-Hernández MM, Rivera-Muñoz G, Gamboa-Angulo M, Sanchez-Teyer F, Couoh-Uicab Y, Canto-Canché B (2018) Fungal screening on olive oil for extracellular triacylglycerol lipases: selection of a *Trichoderma harzianum* strain and genome wide search for the genes. Genes 9:62. https://doi.org/10.3390/genes9020062

Cao X, Liao L, Feng F (2020) Purification and characterization of an extracellular lipase from *Trichosporon* sp. and its application in enrichment of omega-3 polyunsaturated fatty acids. LWT 118:108692. https://doi.org/10.1016/j.lwt.2019.108692

Chandra P, Enespa, Singh R, Arora PK (2020) Microbial lipases and their industrial applications: a comprehensive review. Microb Cell Fact 19:1–42. https://doi.org/10.1186/s12934-020-01428-8

Diaz JCM, Rodríguez JA, Roussos S, Cordova J, Abousalham A, Carriere F, Baratti J (2006) Lipase from the thermotolerant fungus *Rhizopus homothallicus* is more thermostable when produced using solid state fermentation than liquid fermentation procedures. Enzyme Microb Technol 39:1042–1050. https://doi.org/10.1016/j.enzmictec.2006.02.005

El-Ghonemy DH, El-Gamal MS, Tantawy AE, Ali TH (2017) Extracellular alkaline lipase from a novel fungu *Curvularia* sp. DHE 5: optimisation of physicochemical parameters, partial purification and characterisation. Food Technol Biotechnol 55:206–217. https://doi.org/10.17113/ftb.55.02.17.4958

Ezenwelu CO, Afeez OA, Anthony UA, Promise OA, Mmesoma UC, Oparaji E, Henry OE (2022) Studies on properties of lipase produced from *aspergillus* sp. isolated from compost soil. Adv Enzyme Res 10:49–60. https://doi.org/10.4236/aer.2022.102003

Facin BR, Valério A, Bresolin D, Centenaro G, de Oliveira D, Oliveira JV (2018) Improving reuse cycles of *Thermomyces lanuginosus* lipase (NS-40116) by immobilization in flexible polyurethane. Biocatal Biotransfor 36:372–380. https://doi.org/10.1080/10242422.2018.1458842

Fickers P, Nicaud JM, Gaillardin C, Destain J, Thornart P (2004) Carbon and nitrogen sources modulated lipase production in the yeast *Yarrowia Lipolytica*. J Appl Microbiol 96:742–749. https://doi.org/10.1111/j.1365-2672.2004.02190.x

Hafez RM, Abdel-Rahman TMA, Atef A, Abed NN (2024) Effect of some mutagenic compounds on lipase production by fluconazole-treated *Candida catenulata*. Biomass Conv Bioref 14:6237–6250. https://doi.org/10.1007/s13399-022-02737-1

Haki GD, Rahshit SK (2003) Developments in industrially important thermostable enzymes: a review. Bioresour Technol 89:17–34. https://doi.org/10.1016/s0960-8524(03)00033-6

Hölker U, Höfer M, Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl

Microbiol Biotechnol 64:175–186. https://doi.org/10.1007/s00253-003-1504-3
Jaeger KE, Dijkstra BW, Reetz MR (1999) Bacterial biocatalysts: molecular biology, three dimensional structures, and biotechnological applications of lipases. Annu Rev Microbiol 53:315–351. https://doi.org/10.1146/annurev.micro.53.1.315
Ktata A, Bezzine S, Sayari A, Karray A (2020) Newly isolated lipolytic and oleaginous fungal strain, production, optimization and biochemical characterization of the extracellular (phospho)lipase. Waste Biomass Valor 11:6677–6687. https://doi.org/10.1007/s12649-019-00907-3
Kumar A, Verma V, Dubey VK, Srivastava A, Garg SK, Singh VP, Arora PK (2023) Industrial applications of fungal lipases: a review. Front Microbiol 14:1142536. https://doi.org/10.3389/fmicb.2023.1142536
Licia MP, Cintia MR, Mario DB, Guillermo RC (2006) Catalytic properties of lipase extracts from *Aspergillus Niger*. Food Technol Biotechnol 44:247–252
Mahfoudhi A, Benmabrouk S, Fendri A, Sayari A (2022) Fungal lipases as biocatalysts: a promising platform in several industrial biotechnology applications. Biotechnol Bioeng 119:3370–3392. https://doi.org/10.1002/bit.28245
Maia MMD, Morais MMC, Morais MA, Melo EHM (1999) Production of extracellular lipase by the phytopathogenic fungus *Fusarium Solani* fs1. Revista De Microbiol 30:304–309. https://doi.org/10.1590/S000137141999000400003
Margesin R, Gander S, Zacke G, Gounot AM, Schinner F (2003) Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. Extremophiles 7:451–458. https://doi.org/10.1007/s00792-003-0347-2
Mehta A, Bodh U, Gupta R (2017) Fungal lipases: a review. J Biotech Res 8:58–77
Mendes DB, Da Silva FF, Guarda PM, Almeida AF, de Oliveira DP, Morais PB, Guarda EA (2019) Lipolytic enzymes with hydrolytic and esterification activities produced by filamentous fungi isolated from decomposition leaves in an aquatic environment. Enzyme Res 2019: 8182425. https://doi.org/10.1155/2019/8182425
Obradors N, Montesinos JL, Valero F, Lafuente FJ, Solå C (1993) Effects of different fatty acids in lipase production by *Candida rugosa*. Biotechnol Lett 15:357–360. https://doi.org/10.1007/BF00128276
Oliveira GF, Milena RL, Silva MRL, Hirata DB (2021) Production of new lipase from *Preussia africana* and partial characterization. Prep Biochem Biotechnol 52:942–949. https://doi.org/10.1080/10826068.2021.2012684
Panuthai T, Sihanonth P, Piapukiew J, Sooksai S, Sangvanich P, Aphichart Karnchanatat A (2012) An extracellular lipase from the endophytic fungi *Fusarium oxysporum* isolated from the Thai medicinal plant, *Croton oblongifolius* Roxb. Afr J Microbiol Res 6:2622–
Qu P, Li D, Lazim R, Xu R, Xiao D, Wang F, Li X, Zhang Y (2022) Improved thermostability of *Thermomyces lanuginosus* lipase by molecular dynamics simulation and *in silico* mutation prediction and its application in biodiesel production., Fuel 327: 125039. https://doi.org/10.1016/j.fuel.2022.125039
Rai B, Shrestha A, Sharma S, Joshi J (2014) Screening, optimization and process scale up for pilot scale production of lipase by *Aspergillus niger*. Biomed Biotechnol 2:54–59. https://doi.org/10.12691/bb-2-3-3
Rajendran A, Palanisamy A, Thangavelu V (2008) Evaluation of medium components by Plackett-Burman statistical design for lipase production *by Candida rugosa* and kinetic modeling. Chin J Biotech 24:436–444. https://doi.org/10.1016/S1872-2075(08)60024-2
Salihu A, Alam MZ, Abdul Karim MI, Salleh HM (2011) Suitability of using palm oil mill effluent as a medium for lipase production. Afric J Biotechnol 10:2044–2052
Sharma R, Chisti Y, Banerjee UC (2001) Production, purification, characterization, and applications of lipases. Biotechnol Adv 19:627–662. https://doi.org/10.1016/s0734-9750(01)00086-6
Sierra G (1957) A simple method for detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonine Van Leeuwenhoech 23:15–22
Stránský K, Zarevùcka M, Kejík Z, Wimmer Z, Macková M, Demnerová K (2007) Substrate specificity, regioselectivity and hydrolytic activity of lipases activated from *Geotrichum* Sp. Biochem Eng J 34:209–216. https://doi.org/10.1016/j.bej.2006.12.006
Suryanarayanan TS, Govindarajulu MB, Thirumalai E, Reddy MS, Money NP (2011) Agni's fungi: heat–resistant spores from the western ghats, southern India. Fungal Biol 115:833–838. https://doi.org/10.1016/j.funbio.2011.06.011
Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120
Tsuzuki W, Kitamura Y, Suzuki T, Mase T (1999) Effects of glucose on lipase activity. Biosci Biotechnol Biochem 63:1467–1470. https://doi.org/10.1271/bbb.63.1467
Van Kan JAL, van den Ackerveken GF, de Wit PJ (1991) Cloning and characterization of the cDNA of avirulence avr9 of the fungal pathogen *Cladosporium Fulvum*, the causal agent of tomato leaf mold. Mol Plant Microbe Interact 4:52–59. https://doi.org/10.1094/mpmi-4-052
Verma N, Thakurm S, Bhatt AK (2012) Microbial lipases: industrial applications and properties. Int J Biol Sci 1:88–92
Vijayan AK, Abin V, Sumit SS, Mahesh M (2020) Impact of forest fire in the Nilgiri Biosphere Reserve, Western Ghats – A geoinformatics based analysis. Clim Change Environ Sustain 8:24–37. https://doi.org/10.5958/2320-642X.2020.00003.4
Yadav R, Vasundhara M, Rajamani T, Suryanarayanan TS, Reddy MS (2022) Isolation and characterization of thermostable and alkalitolerant cellulase from litter endophytic fungus *Bartalinia Pondoensis*. Folia Microbiol 67:955–964. https://doi.org/10.1007/s12223-022-00991-4