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Isolation and Screening of Extracellular Lipase Producing Fungi From Soil

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ABSTRACT

Microbial lipases (extracellular in origin) are the most broadly utilized industrial enzymes. Some industries (detergent, food, oil and paper) demand for lipolytic microbes to fulfill several applications. Therefore, identification and isolation of lipolytic fungi from soil is very significant for such industries. Soil is the best habitat for several microorganisms, secreting enzymes of extracellular origin thus soil samples were obtained from five diverse oil mills of Newai town and were utilized in the current study. Soil samples were processed by serial dilution agar plate method for the isolation of fungi in PDA (Potato dextrose agar) plates. Isolated fungi were further qualitatively evaluated for extracellular lipase production on tributyrin agar (TBA) medium. Lipolytic activity was identified by the formation of halo zone (clear zone of tributyrin degradation) around colonies of fungi. Formation of clear zone around the colonies indicated the production of extracellular lipase, which hydrolyzed the tributyrin and opacity of the medium around such colonies was not retained. Among the all tested thirty one fungal isolates for extracellular lipase production in TBA media, twelve isolates demonstrated zone of hydrolysis. Based on the interpretation of primary screening, four fungal isolates (LPF-5, LPF-9, LPF-17 and LPF-28) were further confirmed for extracellular lipase production in SmF using quantitative method. Among the four isolates, Maximum lipase production (82.21 ± 0.90 U mL^{-1} min^{-1}) was obtained by isolate LPF-5 at 72 h of incubation at 28 ºC.

Key words: Lipase, lipolytic microbes, tributyrin agar medium, zone of hydrolysis, quantitative assay

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INTRODUCTION

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) belonging to the class Hydrolases catalyze hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol [1]. In comparison to esterases, lipases show their catalytic action only after adsorption to a water-oil interface [2]. Lipases are very beneficial biocatalysts for modification of oils and fats due to their capability of catalyzing various reactions other than the hydrolytic reactions [3].

Lipases (and esterases) are capable of catalyzing three types of reactions. The biological action of lipases is reversible. They are capable of catalyzing hydrolysis in an aqueous environment and esterification in a micro aqueous environment, in which content of water is very low. Transesterification is classified into four subclasses (Alcoholysis, acidolysis, interesterification and aminolysis) based on the nature of chemical species which take part in reaction with the ester. There are huge differences between the chemical and lipase-catalyzed reactions. The lipase-catalyzed reactions can be carried on in weaker conditions than the chemical reactions. This is beneficial because unwanted side reactions such as heat degradation of the substrate molecules can be prevented [3, 4]. Lipases are being utilized in different industries such as detergent, agrochemical, paper, chemical, dairy, oleochemical, cosmetics, pharmaceuticals, polymer synthesis, synthesis of surfactants and personal care products [4].

Based on origin, lipases can be categorized into three groups i.e. animal, microbial and plant. Among the all sources of lipases, the most broadly applied lipases were microbial due to their stability and chemical properties [5]. Several organisms such as bacteria, yeasts, actinomycetes and moulds are associated with the production of microbial lipases [6].

Among the all microbial lipase producers, filamentous fungi are preferred sources of lipases. The main producers of commercial lipases mainly are *Rhizopus* sp., *Pencillium* sp., *Aspergillus* sp., *Mucor* sp., *Candida rugosa*, *Acremonium strictum*, *Lipomyces starkeyi*, *Humicola lanuginose*, *Cunninghamella verticillata* and *Geotrichum candidum*. Lipases from *Aspergillus*, *Rhizopus* and *Geotrichum candidum* strains are attractive catalysts for lipid modification [7].

Lipolytic microorganisms have been isolated from diverse habitats such as edible oil extraction factories, diesel and edible oil contaminated soil, industrial wastes, dairies, decomposed food and oilseeds, coal tips, hot springs and compost heaps [8]. Although a number of publications are available on well-characterized lipolytic microorganisms, still there is an interest in finding new sources of lipase for various applications. Soil is one of the best sources for the isolation of novel

lipase producing microorganisms as it gives nutrient rich environment to enable the high proliferation of microorganisms. The diversity of such microorganisms depends on several physio-chemical properties related to the climate and type of soil viz. pH, texture, solar radiation, temperature, aeration, mineral composition and water contents [9]. Presently, the demand of the industries for novel sources of lipases with diverse catalytic features promotes the isolation and screening of novel lipolytic microbial strains [10]. Extensive work on lipase producing fungi is available with main emphasis on cloning and expression of lipase producing gene, determination of three dimensional structure of lipase, kinetic parameters, and enzyme action [11]. In contrast, comparatively few works has been done on screening of lipolytic fungi from edible oil contaminated soil. The goal of present investigation was to identify and isolate potent lipolytic fungi from soil samples. To achieve this object, edible oil contaminated soil samples collected from five different oil mills of Newai town were used for the isolation of fungi on PDA plates. Nearly 30 oil mills are located in Newai producing edible oil from Groundnut, Mustard, Soyabean, and Taramira seed grains. Due to improper management of effluent disposal, adjoining areas are heavily contaminated with leakage and waste from these industries.

MATERIALS AND METHOD

Sample collection:

Soil samples were taken from the five different oil mills of Newai town (Table 1). Town Newai (Tonk district) is situated in southeast Rajasthan. The region lies between 26°10′ North latitude to 75°47′ East longitude. Soil samples were collected arbitrarily from surface layer of soil by spatula and taken in sterile Petri plates, tagged with date and place of collection. Samples were stored in freeze at 4 ºC till their use [12].

Isolation of fungi:

Fungi were isolated by serial dilution agar plate method as described earlier by Waksman [13]. Initial dilution (10^{-1}) was made by adding one gram of soil in 9 mL of autoclaved distilled water followed by vigorously shaking. Next dilution (10^{-2}) was prepared by transferring one mL of the previous dilution in next test tube containing 9 mL distilled water followed by mixing the contents. This procedure was continued up to 10^{-6} dilution. Dilutions were made in laminar air flow chamber. Each of the dilution (0.1 mL) was poured on PDA plates, uniformly distributed by sterile spreader and plates were kept at 28 °C for 7 days [14, 15]. Fungi were detected at the end of incubation and maintained on PDA slants. To isolate fungi, streptomycin was added at a rate of 25 mg/100 mL to the medium [16].

Qualitative screening of lipolytic fungi on tributyrin agar medium (TBA):

Isolated fungi were evaluated for lipase production in TBA plates. The composition of TBA medium (g L^{-1}) is as follows: peptone, 5; yeast extract, 3; agar, 15; tributyrin (glycerol tributyrate), 10 and pH was adjusted to 7.5. Each fungal isolate was point inoculated in the centre of TBA medium plates followed by incubation at 28 ˚C for 7-10 days. Clear zones were observed in the close vicinity of fungal colonies at 7 days of incubation due to hydrolysis of glycerol tributyrate in butyric acid and glycerol [17].

Quantitative screening of extracellular lipase in SmF:

Inoculum preparation:

Seven days old fungal culture already grown in a Petri dish was used for preparation of inoculum. The spores were scratched with sterile inoculating needle and mixed with 5 mL of sterile distilled water in a tube. The contents of tube were shaken vigorously to prepare the uniform suspension of spores. The above suspension of spores was utilized as source of inoculum [18].

Table 1: Sample type, location and sample code number

S. No.	Sample type	Location	Sample code
1	Contaminated with groundnut and mustard oil	Todwal Udyog, Industrial Area, Newai	T
2	Contaminated with groundnut and mustard oil	Suresh oil mill, Jamat, Newai	S
3	Contaminated with groundnut and mustard oil	Kailash Udyog, Industrial Area, Newai	K
4	Contaminated with groundnut and mustard oil	Shri Mahaveer Oil Industry, Behind old police station, Newai	SM
5	Contaminated with mustard oil	Mangalam Protein Pvt. Ltd., Baroni, Newai	M

Transferring spore suspension in production medium:

From the above spore suspension, one mL was aseptically added in 250 mL Erlenmeyer flask containing 100 mL of fermentation broth followed by incubation of flasks at 28 ˚C, 150 rpm for 6 days. The composition of fermentation broth (g L^{-1}) is as follows: bacteriological peptone, 20; olive oil, 1%; $MgSO_4.7H_2O$, 0.6; KH_2PO_4, 1.0; NH_4NO_3, 1.0 and pH was adjusted to 7.0. Agitated condition for culture proved to be more effective and promote lipase production than static culture as culturing in shaking condition provided a better homogenized carbon source (more oil water interface) in the media for the fungi to utilize. And shaking condition also discouraged the formation of spores on the media surface [19].

Recovery of lipase from culture broth:

Upon completion of incubation, culture broth was filtered through Whatmann No. 1 filter paper followed by centrifugation of filtrates at 7,000 rpm, 4 ˚C for 10 minutes. Clear supernatants (crude protein lysate) were obtained after centrifugation and utilized for estimation of lipase activity. The supernatant of crude enzyme was utilized for further investigations [20].

Lipase assay:

Activity of lipase was determined by procedure as described previously by Winkler and Stuckmann [21]. For the preparation of *p*-nitrophenyl palmitate (*p*NPP) stock solution (20 mM), 0.151 g of *p*NPP was dissolved in 20 mL of isopropanol. The reaction mixture was containing 75 µL of *p*NPP stock solution in 2.9 mL of Tris-HCl Buffer (0.05 M, pH-8.0) was preincubated at 70 °C in a water bath shaker for 10 minutes and then 25 µL enzyme (Crude protein extract) was added to adjust the final volume of reaction mixture to 3 mL. The lipase catalyzed reaction was allowed to conduct at 35 °C for 30 minutes in a water bath shaker. Further lipase reaction was terminated by the addition of 1 mL of chilled stop reagent (acetone and alcohol were mixed in equal volume and placed at -20 °C). In control (blank) set, the reaction mixture was incubated without addition of enzyme and 25 µL of enzyme was added after addition of stop reagent (acetone: alcohol, 1:1, v/v). The concentration of liberated *p*-nitrophenol in the reaction mixture was determined at 410 nm against a reagent blank by using standard curve of *p*-nitrophenol, which was prepared in 0.05 M Tris HCl buffer, pH-8.0 within the concentration range of 2-20 µg/mL [22]. Each of the assay was performed in triplicates and their mean values were presented.

One unit (U) of enzyme activity was equivalent to how many micromoles (µM) of *p*- nitrophenol liberated by hydrolysis of ester linkage of *p*NPP by one mL of soluble enzyme at 35 °C in one minute of reaction.

RESULTS AND DISCUSSION

Isolation and screening of lipolytic fungi:

The edible oil contaminated soil samples obtained from five different oil mills of Newai town were used for the isolation of lipolytic fungi. Serial dilution agar plate technique was utilized for isolation of fungi (Figure 1). The isolated fungi were maintained on PDA slants and used for the screening of extracellular lipase production [23]. Results of isolation are presented in Table 2. Higher load of fungi was detected in soil sample “T” which was contaminated with groundnut and mustard oil collected from Todwal Udyog, Industrial Area, Newai and lowest load of fungi was found in soil sample “M” which was contaminated with only mustard oil collected from Suresh oil mill, Jamat, Newai.

Table 2: Number of fungal colonies appeared on PDA plates isolated from five different oil mills of Newai town.

Dilutions	Sample Code				
	T	S	K	SM	M
10^{-1}	TNTC	25	23	TNTC	21
10^{-2}	13	8	12	11	9
10^{-3}	10	7	6	8	5
10^{-4}	5	4	4	5	5
10^{-5}	4	2	3	3	3
10^{-6}	2	Nil	Nil	2	1

Zhou et al. [24] reported isolation of lipolytic fungus *A. oryzae* from oily soil samples. Rai et al. [3] isolated four lipolytic strains of the *A. niger* from oil contaminates soil samples. Colla et al. [25] reported isolation of lipolytic filamentous fungus of *Aspergillus* sp. from diesel contaminated soil samples.

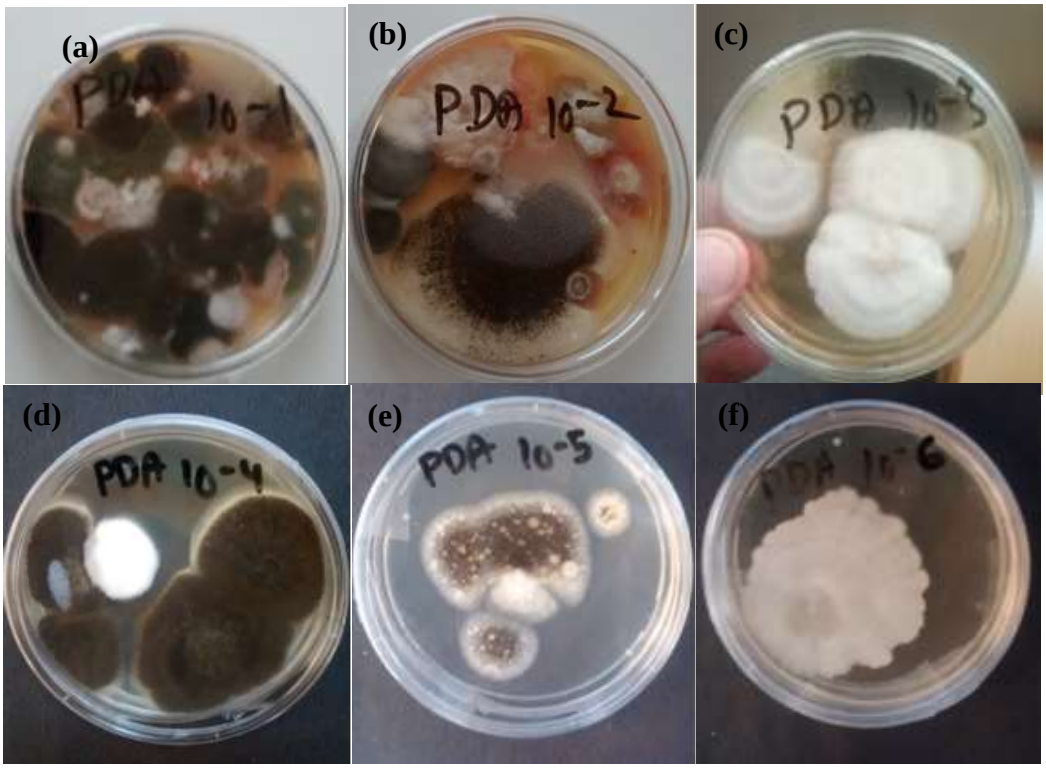
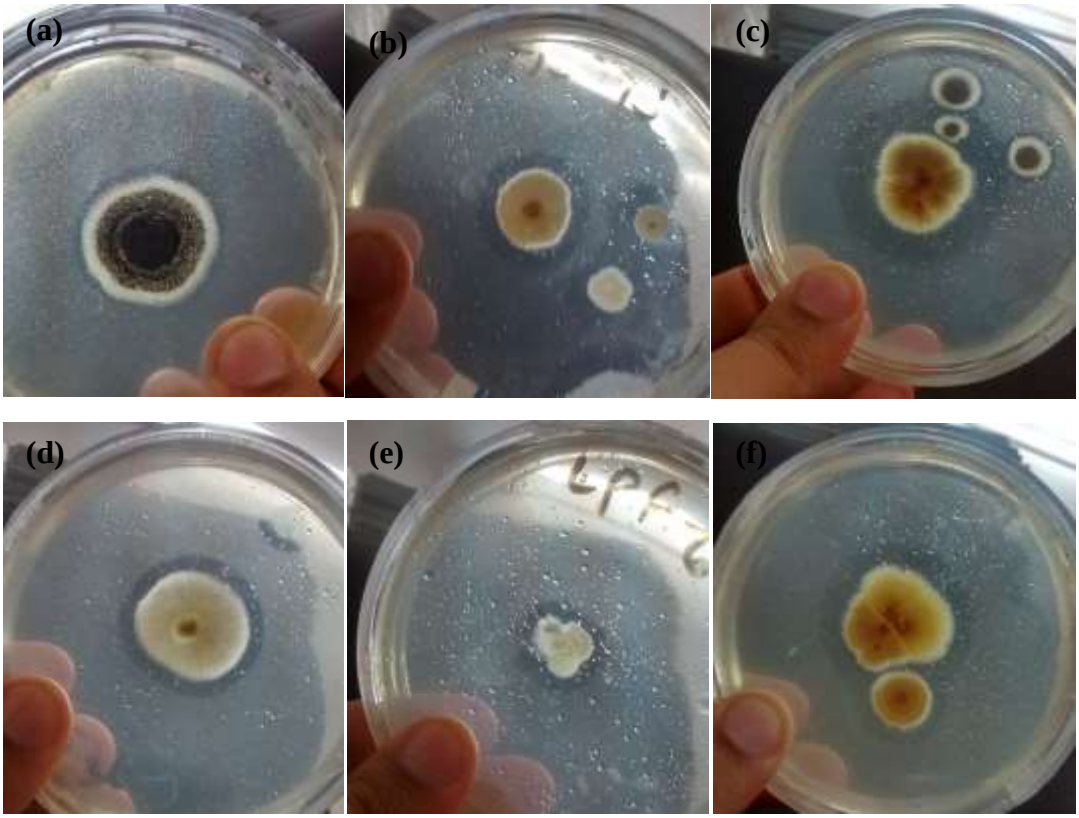


Figure 1: Fungi isolated on PDA plates from different dilutions of soil sample; (a): Dilution 10^{-1}; (b): Dilution 10^{-2}; (c): Dilution 10^{-3}; (d): Dilution 10^{-4}; (e): Dilution 10^{-5}; (f): Dilution 10^{-6}.

The lipolytic activities of fungal isolates were evaluated using TBA medium by the method as suggested by Sarkar et al. [26]. Lipolytic activity was exhibited by the development of halo zone (clear zone of tributyrin degradation) around fungal colonies. Development of clear halo zone around the colonies, indicated the production of lipase of extracellular origin, which hydrolyzed

the tributyrin and opacity of the medium around such colonies was not retained (Figure 2). If agar medium retained opaqueness around fungal colony, this indicated that the organism did not produce extracellular lipase. Among the all tested isolates (thirty one fungal isolates) for extracellular lipase production in TBA media, twelve isolates (38%) exhibited zone of hydrolysis indicating that these twelve isolates produced extracellular lipase which hydrolyzed the tributyrin in their vicinity resulting in the formation of clear zones around their colonies. 10% of all the isolated fungal strains did not exhibit any growth in TBA, which showed that they were unable to utilize any ingredients of TBA medium. Remaining 52% could grow but did not show the zone of lipolytic activity suggesting that they did not produce any extracellular enzyme for tributyrin degradation (Table 3).



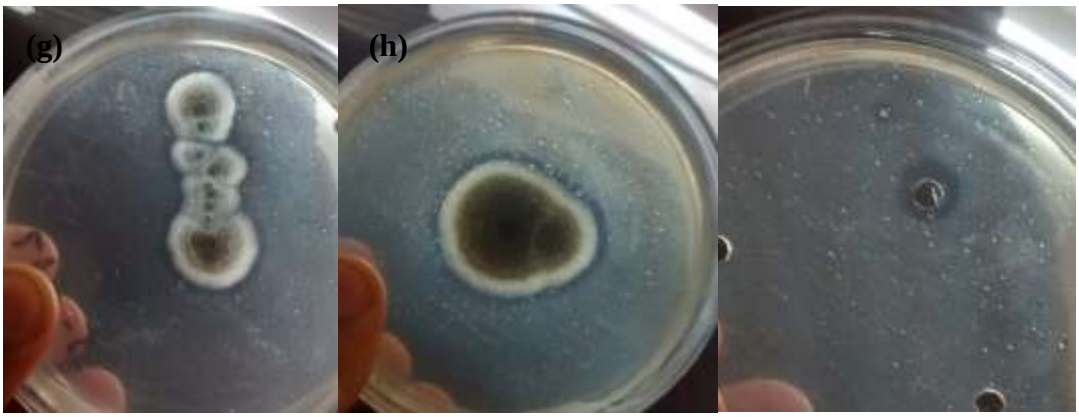


Figure 2: Qualitative screening of fungal isolates for extracellular lipase activity in TBA plates; Zone of hydrolysis by fungal isolate (a): LPF-6; (b): LPF-8; (c): LPF-3; (d): LPF-17; (e): LPF-28; (f): LPF-20; (g): LPF-12; (h): LPF-5; (i): Zone of hydrolysis in TBA gel diffusion assay.

Table 3 Qualitative screening of thirty one fungal isolates for extracellular lipase production in TBA medium

Sample code name	Isolate	Zone of hydrolysis on Tributyrin agar medium
T	LPF-1	-
	LPF-2	-
	LPF-3	+
	LPF-4	-
	LPF-5	+
	LPF-6	+
	LPF-7	-
	LPF-8	+
S	LPF-9	+
	LPF-10	-
	LPF-11	-
	LPF-12	+
	LPF-13	+
K	LPF-14	NG
	LPF-15	+
	LPF-16	-
	LPF-17	+
	LPF-18	-
	LPF-19	-
	LPF-20	+
	LPF-21	-
SM	LPF-22	-
	LPF-23	NG
	LPF-24	+
	LPF-25	-
M	LPF-26	-
	LPF-27	-

LPF-28	+
LPF-29	-
LPF-30	NG
LPF-31	-

+, positive, -, negative, NG, No growth; LPF: Lipase producing fungi

Table 4: Quantitative screening of selected fungal isolates for extracellular lipase production in SmF

Soil sample Code	Fungal isolates	Lipase activity (U mL^{-1} min^{-1})
T	LPF-5	82.21 ± 0.90
S	LPF-9	25.39 ± 0.31
K	LPF-17	67.70 ± 1.20
M	LPF-28	54.40 ± 0.83

Similar to our results, Bapiraju et al. [8] reported that nine isolates revealed lipolytic activities among the thirty four fungi obtained from oily soil samples and examined for extracellular lipase activity. Ravindranath and Lakshmi [27] recently reported development of zone of hydrolysis in TBA medium by *Aspergillus* sp. TL-12 isolated from oily soil samples. Bhavani et al. [28] reported isolation and screening of novel lipolytic bacterial isolates from soil samples. Ulker et al. [29] described qualitative and quantitative screening for extracellular lipase production by a novel fungal strain (*Trichoderma harzianum*) of soil sample. Zhou et al. [24] reported screening of lipolytic fungi from the collected oily waste soil samples. Toscano et al. [6] also reported screening of 12 fungi belonging to genera *Trichoderma, Aspergillus*, *Mucor,* and *Pencilillium* on TBA medium for lipolytic potential.

Quantitative screening of extracellular lipase production in SmF (Submerged fermentation):

The goal of the present investigation was to choose the fungal isolates with elevated potential of extracellular lipase production. To accomplish this aim, a total of 4 fungal strains were quantitatively tested for lipase production in SmF. Lipase activity was estimated after three days of incubation. Activity of lipase was detected in the range of 25.39 ± 0.31 U mL^{-1} min^{-1} to 82.21 ± 0.90 U mL^{-1} min^{-1}. Highest lipase activity (82.21 ± 0.90 U mL^{-1} min^{-1}) was detected in isolate LPF-5 at 72 h of incubation among the all four strains. This hyperproducer strain was isolated from groundnut and mustard oil contaminated soil of Todwal Udyog, Industrial Area, Newai. Isolate LPF-9 demonstrated the lowest lipase activity 25.39 ± 0.31 U mL^{-1} min^{-1} (Table 4). Although Isolate LPF-5 did not demonstrate maximum diameter of halo zone during primary screening in TBA medium but in quantitative screening but it produced highest amount of lipase

than other isolates. It indicates that secondary screening helps in sorting out the microbial strain with real commercial value.

Similar to our results, Naz and Jadhav [30] reported that diameter of halo zones for *A. paraciticus*, *A. niger* and *A. aculeatus* in TBA plates were 6.0, 5.0 and 1.0 mm, respectively but highest lipase activity (2.7 U mL^{-1}) was obtained from *A. niger* followed by *A. paraciticus* (2.5 U mL^{-1}) and *A. aculeatus* (2.7 U mL^{-1}). Rifaat et al. [31] reported higher activity (16.0 U mL^{-1}) of lipase by *Fusarium oxysporum* in SmF. Therefore it was selected for physicochemical studies. Samad et al. [32] reported higher lipase production (23 U mL^{-1} min^{-1}) by a new strain of *Rhizopus rhizopodigormis* at 72 h of incubation. Toscano et al. [6] have reported quantitative screening of twelve fungal strains belonging to different genera for extracellular lipase production. Among the all fungi, maximum activity of lipase (7.5 U cm^{-3}) was obtained from *A. niger*. Maia et al. [33] reported higher lipase activity (9500 U L^{-1}) by *F. solani* FS1 culture at 4 days of incubation in the broth having olive oil (1%, v/v). Costa et al. [34] reported screening of fifty six soil filamentous fungal isolates for extracellular lipase production. Maximum lipase activity was achieved by *Penicillium wortmanii* after seven days of incubation.

CONCLUSION

The core objectives of this research were to isolate potent novel lipolytic fungal strains from edible oil contaminated soil. Thus, soil samples were taken from five different oil mills of Newai. Thereafter, serial dilution agar plate technique was utilized for the isolation of different fungi. A total of thirty one fungal isolates were used for qualitative screening in TBA medium, and twelve isolates exhibited halo zone (clear zone of hydrolysis) around their colonies. Among the twelve lipase producing isolates, only four isolates (LPF-5, LPF-9, LPF-17 and LPF-28) were further selected for quantitative estimation of extracellular lipase in SmF. Maximum activity (82.21 ± 0.90 U mL^{-1} min^{-1}) of lipase was obtained by isolate LPF-5 at 72 h of incubation at 28 ºC. The fungal isolate (LPF-5) that demonstrated significant lipase activity can be purified and characterized further for utilization in industries.

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