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ISOLATION AND CHARACTERIZATION OF FUNGAL LIPASE STRAINFROM OIL SPILLED SOILS

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Abstract:

A total of 17pure fungal lipolytic strains were isolated from five vegetable oil contaminated soil samples collected from five oil mills located in different areas of Guntur district, Andhra Pradesh, India. The fungal isolates were screened for the production of lipase enzyme, qualitatively using Tributyrin Agar medium and Olive oil -Rhodamine-B agar media. Out of the 17 isolated, two strains (rm-104 and rm-125) showed high lipase activity with large hydrolysis zones, were identified based on their morphological characteristics as *Penicillium* sp. These two strains produced 0.155±0.007 $IUmL^{-1}$ and 0.105±0.002 $IUmL^{-1}$ of enzyme at pH 6 and 37°C ± 2°Crespectively in the basal medium. The strain with high enzyme production, rm 104, was identified as ***Penicillium citrinum*** basing on the 18s rRNA sequencing data, and the sequence was deposited in gene bank with accession number **KU613360.**Thus, this straincan be considered as good source for extracellular lipase production under slightly acidic conditions.

Keywords: Isolation, Extracellularlipase, screening, medium.

1.Introduction:

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are the potent class of serine hydrolases that can catalyze triglycerides to mono or diglycerides, glycerols and fattyacids under natural conditions[1].Lipases have a broad platform of substrate specificity by which they can operate on variety of glycerides and non-glycerides esters.The ability and capability of lipases, to form a diverse group of synthetic and hydrolytic products in both aqueous and non-aqueous medium, was considered inmultifold applications in a wide range of industrial bio catalysis processes and bioremediation technology [2-3].

Many reports have published already stating the presence and isolation of lipase producing microbes from a wide range of different zone of environments. Soil contaminated with oils serves as the best source for screening and isolation of diverse lipase producing microbes[4]. Amongst the different microorganisms identified as source of lipase, filamentous fungi are believed as the excellent sources of extracellular lipase for mass production of industrial level. Soil contaminated with spillage from the products of oil and dairy harbors fungal species, which have the potential to secrete lipases to degrade fats and oils [5].Many species belonging to *Mucor, Rhizopus, Aspergillus, Geotrichum* and *Pencillium* are broadly indentified as excellent sources of lipase [6-7]. The abundant industrial applications of lipases have stirred interest in isolation of new lipases from novel sources and strong efforts have been concentrated on the engineering of enzyme with specific properties or better performance for industrial applications [8-9].In addition to industrial application, the lipases gained significance in bioremediation processes in varied environments has been well documented [10-11]. Industrial and domestic waste harbour fungal species of greater potential in degrading fats and oils. Besides waste disposal, bioconversions by fungal activities results in the production of vast number of useful substances. Thus, waste can be converted into an eco-friendly resource [5]. Realizing the huge demand and also the economical importance of these extracellular lipolytic fungal enzymes, in different industries, which are expected to further be enhanced in the near future, screening, isolation and investigation were made to search a novel potential fungal strain which can show a promising enhancement in extracellular lipaseand application in various bioremediation processes[12-13].

2.Materials and Methods:

2.1. Soil Sample Collection Sites:

Soil samples were collected from vegetable oil spilled and oil contaminated areas of different oil mills located in and around Guntur District, Andhra Pradesh, India**(Fig 1)**. The soil samples were collected from a depth of 5-10 cm (approximately 10-50 gm) by a large sterile spatula and placed in sterile plastic bags and transported to the laboratory within a minimum period of time.

Fig 1: Five Oil Contaminated Soil Sample collection sites in and around Guntur

2.2. Microbial isolation:

Soil samples were collected from 5 different vegetable oil contaminated areas in and around Guntur. Microorganisms were isolated by serial dilution method, suspending 1.0 gm of sample in 100 ml of sterile distilled water. From 10^{-4} serial dilution, 0.1 ml of soil suspension was spread on PDA plates and the plates were incubated for 3-6 days at room temperature of 28 °C.After incubation, the isolated fungal colonies were sub-cultured on to slants for maintaining the pure cultures of the fungi, for further studies.

2.3. Qualitative screening for lipase activity on Tributyrin Plates and Olive oil RBA plates:

The lipolytic activity of selected strains was detected by the test using tributyrine agar medium, and conformation was done by growing the isolates on rhodamine agar plates. On trybutyrine agar plates, clear hydrolysis surrounding the colony indicates the lypolytic activity of the fungal isolate. The isolated fungi were inoculated at the centre of Tributyrinagar plates maintained at pH 6.5 and were incubated for 3-5 days at room temperature. After incubation, clear zone formed around the colony was observed and the diameter of the zone was measured. In the confirmatory test, growing the isolates on Olive oil -rhodamine B agar (RBA) plates as previously reported by [14]. Formation of orange fluorescent zone around the colony was observed under UV irradiation (350nm) anddiameter of the clear zone formed around the colony was measured. For this, the RBA agar plates were prepared by using the medium composition of 0.001 g of rhodamine B, 0.8 g of nutrient broth, 0.4 g NaCl, 2 g of Olive oil and 1 g of agar in 100 ml. of distilled water at pH8.5. The isolates were inoculated at the centre of the plate and incubated for 3-5 days at room temperature of 28^0C. The main advantage of using Rhodamine B is, it does not inhibit the growth of test microorganism or change its physiological properties [14,15]. Hence this method is useful in screening lipase producing organisms and is widely practiced worldwide [16].

2.4. Lipase production on basal medium:

After qualitative determination of lipase activity on tributyrin agar and Olive oil -rhodamine B agar (RBA)agar medium the organism was cultured in 100 ml of basal medium by following the composition given by Dheeman *et al.*,(2011) [17]with minor modifications. Basal medium composition (g/L): 0.5 g$NaNo_3$, 0.5 g KCl, $MgSO_4$.7 H_2O, 2.0g KH_2PO_4, 1.0g Yeast extract, 1ml 5.0 g peptone and 1ml olive oil added as carbon source, in a 250ml Erlenmeyer conical flask adjusting the pH 6.5 ±0.5 and temperature 37°C ± 2°C. The inoculated flasks were incubated on a rotary shaker subjected to 150 rpm for 3-5 days. The culture was centrifuged at10,000 rpm for 20 min to separate the fungalmycelium from the broth.The culture filtrate was used as enzyme extract for the lipase activity assay.

2.5.Lipase activity assay:

The lipase (titrimetric)assay was performed using olive oil as substrate by measuring the liberated fatty acid as described by Burket *et al.*,(2004) [18]. The reaction mixture (5ml) containing 1 ml olive oil (emulsified with 7% W/V gum Arabic in 50 mM phosphate buffer) pH 6.5 as substrate. The reaction was initiated by adding 1ml of appropriately diluted enzyme extract to the reaction mixture and incubated 15 min at temperature 37°C± 2°C with continuous shaking at 120 rpm [19]. The reaction was stopped by the addition of 15 ml ethanol. The released free fatty acid in the test sample (T) was titrated with 0.05 N NaOH. Control (B) was made with similar conditions without the inoculation of enzyme source. One unit of lipase was defined as the enzyme that liberates 1 µmol of fatty acid per min at 37°C± 2°C, pH 6.5.

$$\text{Lipase activity} = \frac{(T-B) \times 1000 \times 0.05}{30}$$

2.6. Identification of Fungal Strain:

The best lipase producing fungi was tentatively identified by external morphological characters and microscopic characters of the mycelium and sporangiophores and spores. The identification was further confirmed by the 18S rRNA sequencing method carried out by Macrogen, South Korea. The sequence obtained was initially analyzed at National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (Blast) and phylogenetic tree was constructed to identify the isolate [20].

3. Results and Discussion:

3.1. Isolation of Samples from Soil:

Although many fugal lipases have already been well marked and investigated, the search for new lipases from*Penicillium*sp., with greater potentially to interact with various compounds is still ongoing [21, 22].A total of 57 fungi were isolated from five soil samples collected from five different vegetable oil mills **(Fig: 1)**in and around Guntur. **(Table 1).**Among the 5 sites tested, site 2 and 5 harbour more number of fungi than other siteswith 21 and 15 fungal species respectively.The isolated fungi were identified based on morphological characteristics of mycelium, sporangiophore and spore structure as *Penicillium* species.

Table 1: Numberof fungal isolates from each soil sample.

Soil Sample collection sites	Number of Fungal isolates
Sri lakshmi narasimha oil mills	03
Sasi sri Extraction mills	12
Nico Agro oil products Pvt Ltd	21
Namburi Pullarao Oil traders mills	06
Mustard Oil mills	15
Total	57

3.2. Qualitative screening of lipolytic activities of the fungal isolates:

Qualitative test was performed to investigate the hydrolytic activity of the isolated 57 fungal strains.The test was performed by using tributyrine agar medium, and conformation was done by growing the isolates on rhodamine agar plates.Nearly 30% of the strains **(Table 2)**showed lipolytic activity (17 strains degraded Olive oil while 13 strains hydrolysed the tributyrin). **Table 3** shows in detail the substratesdegraded by each isolate, with a qualitative estimation of the degree of hydrolytic activity produced by these strains. Out of 17 fungal strains selected, 6 strains (rm-104, rm-108,rm-117,rm-125,rm -143,rm -152) showed the moderate to strong results on olive oil and tributyrin medium**(Table 3)**.Among them two isolates produced a larger clear zone than the others on trybutyrin plates, indicating higher lipase activity. These two strains **(rm-104 and rm-125)**isolated from samples 2 and 3 produced orange colour, due to the hydrolysis of olive oil substrate. Furthermore, a fluorescent zone on Rhodamine-B agar medium was observed under UV irradiation (350 nm), indicating that these two

strains were able to hydrolyze olive oil/tributyrin**(Table 3).**From the Table 3 data it is evident that two strains named rm-104 isolated from sample 2 and rm-125 isolated from the sample 3 were potent to produce lipase **(Fig.2)**.

Table2:Preliminaryqualitative screening of the isolated fungal strains.

Soil Sample collection site no.	No. of Fungal isolates	No. of Fungal isolates positive on RBA plates	No. of Fungal isolates positive on Tributyrin agar plates
1	3	1	1
2	12	2	1
3	21	7	3
4	6	2	2
5	15	5	6
Total	**57**	**17**	**13**

Tributyrin, is convenient substrate becauseit is easily dispersed in water by shaking or stirring withoutthe addition of any emulsifiers. Tributyrin is a very strongsurface-active substance, and its hydrolysis can be followedby measuring the increase in the diameter of the clear zone.The lipolytic potential of fungi was confirmed bythe Rhodamine method because the enzyme will fluorescewith orange compound as reported by Koukerand Jaeger (1987) [14]. Furthermore, Hou and Johnston (1992) as wellas Lee and Rhee (1993) [23,24][proved that this method is highlysensitive and reliable as a lipase assay. Gopinathe et al (2005) [25] using the rhodamine agar screening method, isolated 14 lipolytic fungi from oil spilled soil samples including *P. citrinum.* Tagore and Narasu (2014) [26] have isolated 150 fungal species from oily soil samples and of which 14 were lypolytic. Similarly, Toshi and Sudhir kumar, 2017 [27] isolated the lipase producing fungi from oil contaminated soils and reported that out of total 15, only five fungi were potent to produce lipase on TBA agar plates including *Penicillium* sp.These reports reveal that oil spill soils are rich sources for diversified lipolytic fungal species.

Table 3: Hydrolytic activitystudies ofthe fungal isolates.

Samples	**No of Fungal Strains Isolated**	**Name of the Strains Selected**	**Temp. ^{0}C**	**Medium**	**Olive Oil - Rhaodamine**		**Tributyrin**
					Colour	**Fluorescence**	
1	3	rm-102	40	PDA	+	+	++
2	12	**rm-104**	32	PDA	++	+++	++++
3	21	rm-117	40	PDA	+	+	++
		rm-125			++	+++	+++
4	6	rm-143	30	PDA	+	++	++
5	15	rm-152	30	PDA	++	+	++
Total	57	6					

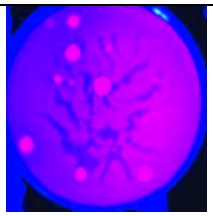
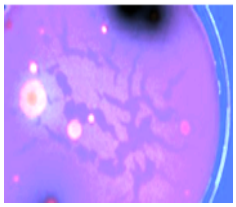
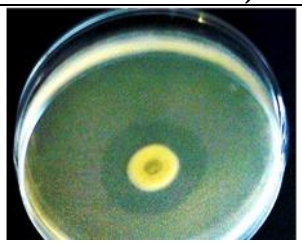
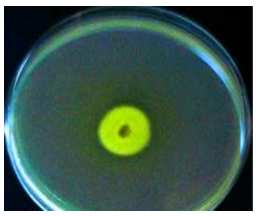
rm-104 strain	rm-125 strain
A) Olive oil –Rhodamine B agar (RBA) plates	
	
B) Tributyrin agar medium	
	

Fig. 2: Hydrolytic activity of rm-104 and rm-125 isolates on Olive oil and tributyrin agar medium

3.3.Morphological Identification of fungal strain:

The above results confirmed that the two strains were potent to produce lipase and also indicate that there are lipolytic fungi. The selected strains (rm-104 and rm-125) were then identified based on morphological characterizations. The morphological characteristics are presented in **Table 4**. The results showed that both strains are spherical shape. Taken together, these characteristics indicated that both strains belong to the genus *Penicillium sp.*

Table 4: Morphological Characters.

Character	rm-104 Strain	rm-125 Strain
Size	0.2-0.5µm	0.2-0.4 µm
Mycelium	Filamentous Branched	Filamentous Branched
Shape	Spherical	Spherical
Sporangiophores	White to Black	White to Black
Growth	Vigorous growth	Confluent growth
Stolons	Present	Present
Aniline dye	Blue	Blue

3.4. Lipase activity on Basal Medium:

Lipase activity was tested forrm-104 and rm-125stains by growing them on basal medium.Maximum lipase activity of 0.155±0.007 $IUmL^{-1}$and 0.105±0.002 $IUmL^{-1}$ was recorded for rm-104and rm-125 strains in the basal medium at pH 6.5 and 37°C ± 2°C,respectively(**Table 5)**.Similar reports on other species of *Penicillium*by Rehaman *et al.*,(2011) [28]on *P.notatum*, Abdullah, (2018) [21]on *Penicillium sps.*, Amin and Bhathi, (2014)[29]on *P.fellutanum*strongly supports our results.

Table 5: Production of extracellular lipolytic activity in the basal medium.

Soil Sample Number	Selected Isolates	Lipase activity ($IUmL^{-1}$)
2	**rm-104**	**0.155±0.007**
3	**rm-125**	**0.105±0.002**

For the identification of the potential fungal strain rm -104, 18S rRNA analysis was done and based on the similarity studies, the isolate showed 97.4% similarity with*Penicillium citrinum*MG711907 strain. The sequence of the identified species was deposited in Genbank with accession number KU613360.**(Fig 3).**

3.5.Molecular Identification of Best Fungal lipase Strain:

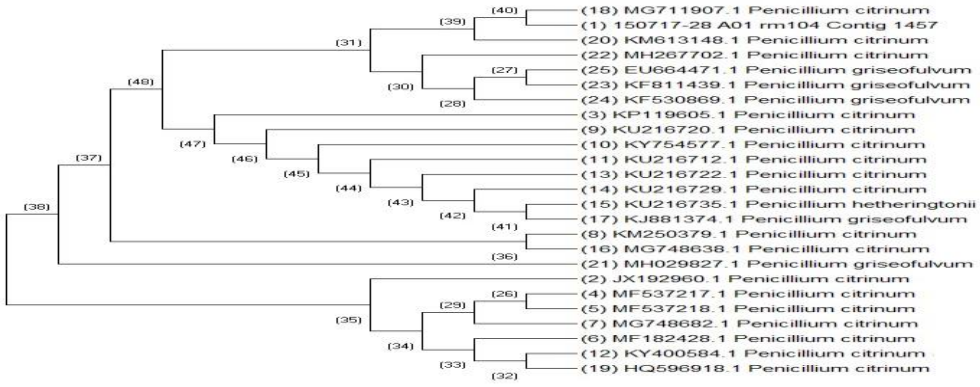


Fig 3: Phylogenetic analysis of rm-104 strain

4.Conclusion:

The frequency of an inducible and extracellular lipase in fungi grown on natural vegetable oilis well documented in lipid biotechnology. A lipase that isstable at acidic conditions and above room temperature ishowever rare, and in the present study we have isolateda novelfungal organism (***Penicillium citrinum*KU613360**) which produces aninducible, extracellular and slightly acidophilic lipase. Therefore, theorganism reported in this paper can be exploited forcommercialization as lipase of these characteristics findsimmense application in various fields of industrial processes.However, further work is clearly needed to increase theproduction of lipase by***Penicillium citrinum* KU613360**wild strain.

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