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Research Article

# SCREENING OF EXTRACELLULAR LIPASE RELEASING MICROORGANISMS ISOLATED FROM SUNFLOWER VEGETABLE OIL CONTAMINATED SOIL FOR BIO-DIESEL PRODUCTION

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**ABSTRACT**

**Objective:** Biological conversions of renewable vegetable oil into bio-diesel are going to become one of the future energy resources for the world.

**Methods:** Soil samples spilled with sunflower oil decomposes, collected from a sunflower oil refinery is used for screening fungal strains. Potato dextrose agar plates were used to isolate, purify the fungal species. Lipase activity of the extracellular lipase enzyme producing fungal species was tested with tween 80, rhodamine-B and phenol red containing agar plates.

**Results:** Five differently colored fungal strains were observed, isolated, purified and compared for their lipolytic activity.

**Conclusion:** A brown colored fungal colony was observed to have the highest lipase activity towards the log chain glycerides.

**Keywords:** Lipase enzyme, *Aspergillus* fungal species, Characterization.

## INTRODUCTION

Energy consumption has become the index of country development. Fossil fuels being depleted, the development of internal renewal energy sources has become very imperative [1]. Agro-based non-edible oils are expected to become one of the main renewal energy resources of the future since the non-edible oils can be chemically converted into bio-diesel - An alternative to the petroleum-derived diesel fuel. The chemical process involving a multi-step hydrolysis of oils in to fatty acids with a subsequent esterification of the fatty acids with methanol or ethanol to an ester has been successfully commercialized in many countries to reduce their oil imports and save their foreign exchange. However, the production is energy intensive and the bio-diesel is also of poor yield and inferior quality compared to the fossil sourced fuels.

The multi-step conversion by hydrolysis [2] cum esterification or a single step trans-esterification processes [3] can be done alternatively with biological enzyme catalysts referred as lipase. Biological conversions are known to be faster giving better yield than chemical reactions [4]. Microorganisms produce both internal and extra cellular lipases with a high level of selectivity and activity. Among them, fungi were the more utilized for industrial applications [5]. But, the cost of production of lipase enzymes as of now is very cost prohibitive and stands in the way of commercialization of the biological method of production of bio-diesel. Hence, attempts to isolate or develop fungal species and optimize the conditions of enzyme production at the lowest cost will be of research interest for some time.

Fungi can be harvested from a wide range of substrates of natural and contaminated soils in the ambient environment [5]. This report details the results of isolation, purification and characterization of a fungal species with relatively high level of hydrolyzing activity from a soil collected in the nearby area of a sun flower edible oil manufacturing plant.

## METHODS

### Sample collection

Few grams of soil samples were collected from different locations of a sun flower oil refinery unit in a sterilized container, mixed together and stored in a dark place at ambient temperature.

### Isolation fungal strains

1.0 g of soil sample was sprinkled aseptically over sterilized plate containing potato dextrose medium (200 g of potato, 20 g of dextrose, 20 g of agar and 1000 ml of distilled $H_2O$ and adjusted to pH 6.6-7.2). Antibiotic - ampicillin was also added aseptically to the sterilized medium to avoid bacterial growth. The plates were incubated at 37°C for 2-4 days and checked every day for growth [6]. A loop of each fungal colony observed in the agar plate was removed successively, serially diluted, incubated at 37°C for 2 days and checked for growth separately. Repeated sub-culturing by streaking on potato dextrose agar (PDA) medium was used to purify the culture. The isolates were maintained on PDA slants at 4°C.

### Morphological identification of fungal isolates

The texture and color of the fungal colonies were observed. Fungal nature of the colonies was confirmed by staining. A drop of distilled water was placed with the help of an inoculums loop on a clean glass slide. The fungal culture was smeared and heat fixed. A drop of 95% ethanol was added to the smear and allowed to evaporate. A drop of lacto-phenol cotton blue stain was added and left for 2 minutes. The stain washed away with distilled water and air-dried. The smear was covered with a clean cover glass. The stained slides were seen under a ×40 objective lens of a binocular light microscope (Labomed, India). The fungal isolates were identified based on the shape of conidia and arrangement of spores on the mycelia [7].

### Screening of lipase producing organisms

Lipase activity can be observed by the formation of a cleared halo rings in agar plates containing appropriate additional ingredients [8].

1. Tween 80: Agar plate is prepared with a medium containing 1.0 g of peptone, 0.5 g of NaCl, 0.01 g of $CaCl_2.5H_2O$, 7 g of $MgSO_4.7H_2O$, 7 g of $KH_2PO_4$, 1.5 g of agar and 1 g of tween 80 in 100 ml of distilled water. After solidification wells can be made in the agar. Stock solutions containing equal number of pores or vegetative fungi of the different stains are added to the separate wells and allowed to incubate at 37°C for 48 h. The diameter of the circular clear region around each well is noted. Larger the ratio, higher is the lipase activity

2. Rhodamine B: Agar plate containing 0.001 g of rhodamine B, 0.8 g of nutrient broth, 0.4 g NaCl, 2 g of olive oil and 1 g agar in 100 ml distilled water adjusted to pH 6.5 is prepared. The stock culture with same concentrations is added to the wells, incubated. Formation of an orange fluorescent zone around the fungal colonies was visible upon ultraviolet (UV) irradiation (350 nm) and the halo diameter measured
3. Phenol red: Agar plates containing 0.01 g phenol red, 0.1 g of $CaCl_2.5H_2O$, 2 g of olive oil and 2 g agar in 100 ml water was prepared. The different strains are incubated in the wells and the halo diameter measured as before.

**Molecular identification of the highly active brown fungal colony [9]**

The molecular tool analysis of the fungi was carried out Chromous Biotech Pvt. Ltd., Bengaluru.

Following are the steps adopted to identify the brown fungi:

1. Extraction of the genomic DNA from the fungal sample
2. The ~700 bp internal transcribed spacer (ITS) region was amplified using a high fidelity polymerase chain reaction (PCR) polymerase
3. Sequencing of the PCR product bi-directionally and
4. Analysis of the sequence obtained for identification of the fungi as well its close neighbor.

The mycelium from the pure culture plates were harvested by filtration through Whatman No.1 filter paper and the genomic DNA from the fungal sample using the fungal genomic DNA isolation kit - RKT13.

PCR amplification, was carried out with:

DNA: 1 µl (100 ng), forward primer: 400 ng, reverse primer: 400 ng, dNTPs (2.5 mM each) 4 µl, ×10 Taq DNA polymerase assay buffer 10 µl, Taq DNA polymerase enzyme (3 U/µl) 1 µl, water X µl to make up the total reaction volume: 100 µl.

The following cycle times were set for the different processes: Initial denaturation at 94°C for 5 minutes, followed by a 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72° for 45 seconds, and a final extension at 72°C for 5 minutes at a final maximum $MgCl_2$ concentration of 1.5 mM. The PCR amplified product was subjected to 1.2% agarose gel (with ethidium bromide) electrophoresis for bp size analysis.

Sequencing of the PCR amplified product was performed on ABI 3500 × L Genetic Analyzer of Applied Bio system Micro Amp, USA, using cycle sequencing kit and using Big Dye Terminator Version 3.1. 10 µl of the sequencing analysis mixture contained 4 µl of Big Dye Terminator Ready Reaction Mix, 1 µl of PCR amplified product (100 ng/µl), 2 µl primer (10 pmol/λ) and 3 µl Milli-Q Water.

Analysis conditions for sequencing was programmed to - denaturation at 96°C for 1 minutes, followed by 25 cycles of denaturation at 96°C for 10 seconds, hybridization at 50°C for 5 seconds and elongation at 60°C for 4 minutes. The resultant nucleotide sequence was analyzed using the software Seq Scape version 5.2, which follows a analysis protocol of BDTv3-KB-Denovo_v 5.2.

Jukes–Cantor corrected distance model was used to generate a distance matrix. A minimum comparable position of 200 ignoring alignment insert was used. The phylogenetic tree was created using Weighbor with alphabet size 4 and length size 1000 using utilizing the sequences aligned with a system software aligner Seq Scape_v 5.2.

## RESULTS AND DISCUSSION

Five differently colored fungal colonies were observed on the soil sprinkled PDA plate (Fig. 1).

Serial dilution culturing of one such white strain is shown in the Fig. 2.

Pure streak cultures of five different colored strains obtained from the sunflower oil processing unit are shown in Fig. 3.

Presence of hyphae observed under microscope in the staining experiment confirmed the microorganism to be of fungal colonies. The pure cultures are tested for their relative lipase activity on agar plates containing tween-80, rodamine-B and phenol red. Tweens are fatty acid ester of polyoxy ethylene sorbitan. The extra cellular lipases produced by the fungi will hydrolyze the ester to liberate the fatty acid. The fatty acids produced around the fungi forms visible opaque crystal of calcium salt as a halo circle around the inoculation well. In rhodamine test, the partially hydrolyzed olive oil products mono and diglycerides forms dimeric complex with rhodamine-B and show fluorescence under UV light at 350 nm. Tween 80, rhodamine-band phenol red showed regions of hydrolysis. Organisms that produce extra cellular lipases can hydrolyze the glycerides. The diameter of the halo regions formed is a measure of the hydrolyzing/lipolytic ability of the colony.

Relatively brown colored strain was showing larger diameter of halo region indicating higher lipase activity (Fig. 4).

**Fig. 1: Sun flower oil contaminated soil potato dextrose agar – Sprinkle plate**

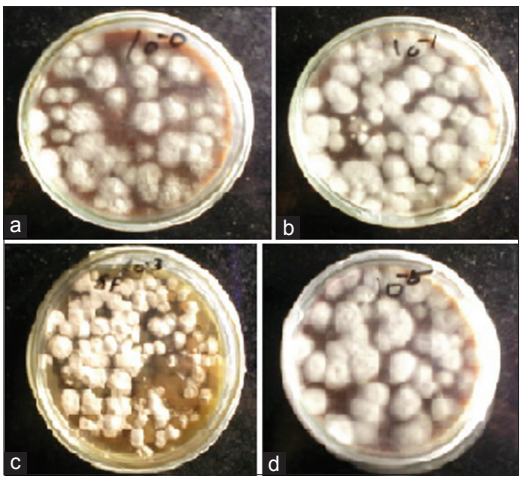


**Fig. 2: Serial dilution – Pour plaes of white coloured colonies, (a) Dilution $10^0$, (b) dilution $10^{-1}$, (c) diltuion $10^{-3}$, (d) diltuion $10^{-5}$**

**Fig. 3: Stains isolated from the sunflower oil contaminated soil**

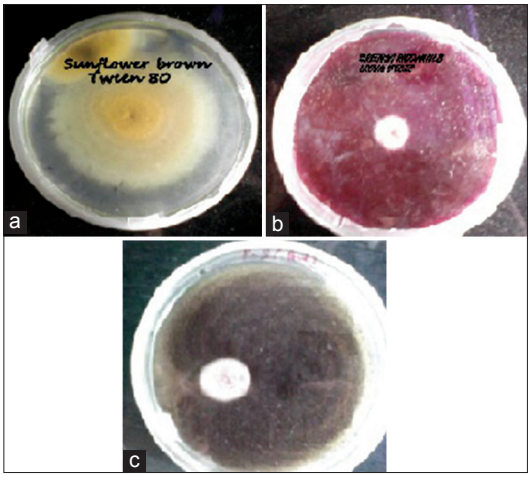


**Fig. 4: Lipase activity of brown colored fungi on agar plates, (a) Tween-80, (b) rhodamine-B, (c) phenol red**

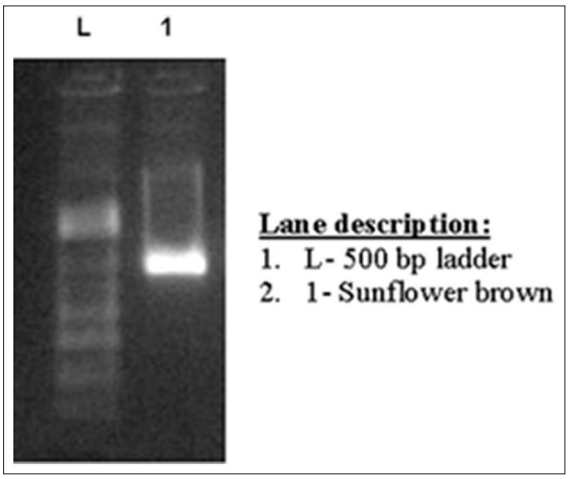


**Fig. 6: Agarose gel eletrophorosis of polymerase chain reaction amplicons of sunflower soil derived brown fungi, lane description: (1) L-500 bp ladder, (2) 1-Sunflower brown**

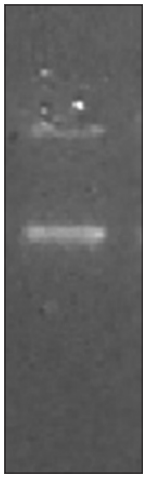

**Fig. 5: Agraose gel electrophorosis of extracted genomic DNA**

2. CTATTGTACCTTGTTGCTTCGGCGGGCCCGCCAGCGTTGCTGGCCGCCGGGGG
54 GCGACTCGCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACATGAACCCTGTT
106 CTGAAAGCTTGCAGTCTGAGTGTGATTCTTTGCAATCAGTTAAAACTTTCAAC
159AATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAC
211 TAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCG
264 CCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAG
317 CCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACGGGCCCGA
369 AAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCTTC
421 CGCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCATTTATTTGCAACTTGTTTT
474 TTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATC
527 AATAGGCAGGAGGACTACCCTCTTTCGCTGTCACATATGGCCCAACCTCCCA
579 CCCGTGACTATTGTACCTTGTTGCTTCGGCGGGCCCGCCAGCGTTGCTGGCCG
632 CCGGGGGGCGACTCGCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACATGAA
684 CCCTGTTCTGAAAGCTTGCAGTCTGAGTGTGATTCTTTGCAATCAGTTAAAAC
737 TTTCAACAATGGATCTCTTGGTT

**Fig. 7: 750 bp internal transcribed spacer region nucleotide sequence of the active brown fungi**

Hence brown colored strain was taken for identification.

The agarose gel-electro-phorosis analysis of the extracted genomic DNA is shown in the Fig. 5.

Agarose gel analysis of the PCR amplified product is shown in Fig. 6.

The ITS region nucleotide sequence of the analyzed brown fungi is shown in Fig. 7.

The phylogenetic tree analysis is drawn in Fig. 8.

The fungal strain was identified as belonging to *Aspergillus* species being closer to *Aspergillus terreus* isolate PKU F22 18S and a next closest homologue as *Aspergillus tubingenesis* strain ATU-KSU09 internal transcribed spacer 1.

## CONCLUSION

We are interested in the screening of extra cellular lipase producing fungal strain, for biodiesel production. Soil samples were collected from sunflower oil processing refinery and used to isolated five strains. They were sub-cultured to purify and tested for their lipase activity on tween-80 and olive oil. A brown colored strain was showing relatively higher hydrolysis of the fatty ester. The brown strain was identified to be of *Aspergillus*, closest to *A. terreus* isolate PKU F22 18S ribosomal RNA gene (NCBI Acc. No: gb/KC113303.1) and the next closest homologue being *A. tubengenesis* strain ATU-KSU09 internal transcribed spacer 1 (NCBI Acc. No: gb/HM753602.1).

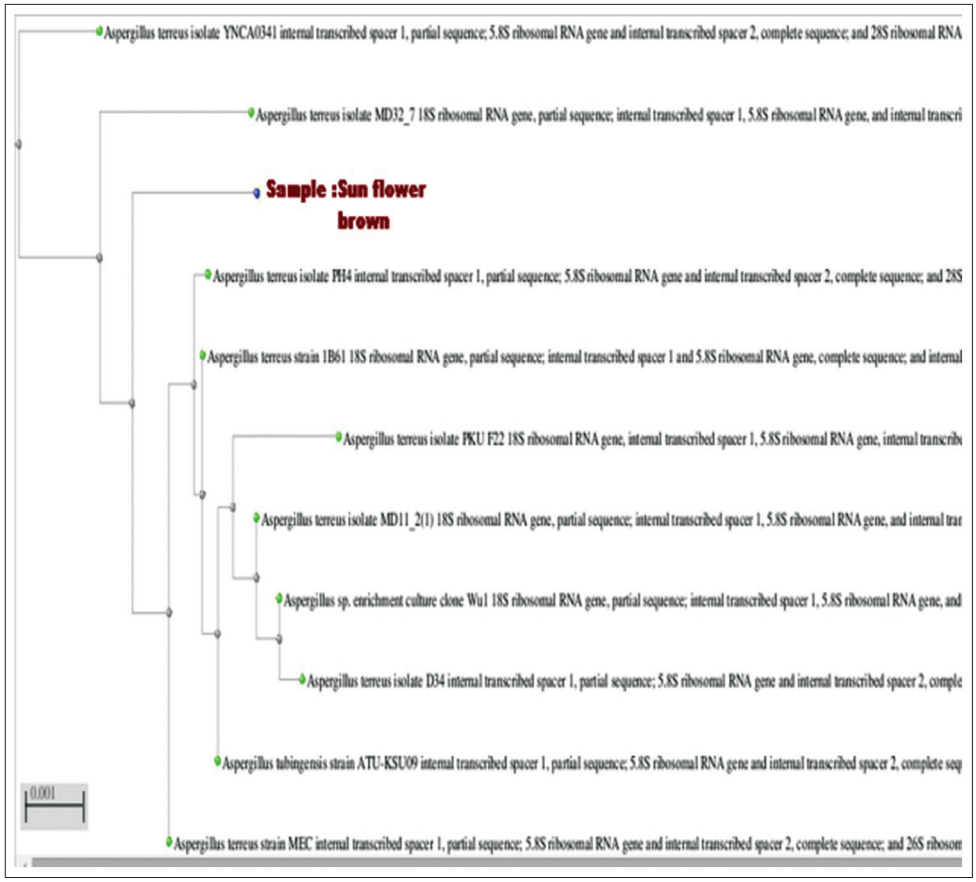


**Fig. 8: The phylogenetic tree showing the relationship of the brown strain with other related species in the NCBI database**

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