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SCREENING OF MICROORGANISMS ISOLATED FROM PETROLEUM OIL CONTAMINATED SOIL FOR VEGETABLE OIL CONVERSION TO BIO-DIESEL

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ABSTRACT

Decreasing fossil fuel stocks has prompted research and development to find alternatives, particularly from renewable energy resources. Lower alcohol, fatty ester called as Bio-Diesel production using vegetable oils with the help of biological catalysts (Lipases) is being considered as one such good alternative. Soil samples in and around petroleum stock processing plant wereused to isolate fungal species. Fungal species were purified on PDA agar plates and tested for their relative ability to hydrolyze glycerides. The stain that showing high level of lipase activity on Tween 80, Rhodamine-B and Phenol red containing agar plates was selected and characterized. Three differently colored and fast growing fungal colonies were observed, isolated, purified and tested for their relative lipolytic activity. Black colored fungal colony was observed to have highest lipase activity in both tween-80 and Rhodamine-B agar plates for fatty ester and olive oil. The sample was found to be *Aspergillus niger* strain, resembling closely WHAK1 18S ribosomal RNA gene. The nucleotide sequence is annotated with NCBI accession no. KR261667

KEY WORDS: **Lipase enzyme, *Aspergillus niger*, agarose electrophoresis, identification.**

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INTRODUCTION

Fossil fuel resources are fast depleting. Development of alternate renewal energy sources is very important in the energy intensive modern world. Vegetable non edible oils resources are untapped renewal resources that can be converted into fuel alternatives called as BIO-DIESEL[1]. The process is of much economical importance for agricultural based countries like India. The vegetable oils (both edible and non-edible) can be hydrolyzed with bases to fatty acids which can be subsequently esterified with methanol or ethanol in the presence of acid chemical catalysts. The oil ester is called as BIO-DIESEL. The chemical process technology is well established and commercialized in many countries successfully[2]. But the chemical conversion is a multi step process, involving large energy, downstream treatment, and effluent disposal and apart produces an inferior quality Bio-diesel. Biological conversions are known to be highly active, more selective, less energy intensive and involve easy biodegradability[3]. Lipase enzyme produced by microorganisms ound to be capable of catalyzing both hydrolysis[4] and esterification as of in a multi step process or to trans-esterify [5] in a single step the vegetable oils into bio diesel [6]. But the production of the lipase enzyme and its activity differs from species to species and conditions of production. Identification of highly active and cost effective lipase is critical for the commercialization of biological production of biodiesel. Fungi were considered to be the best lipase sources and are preferably used for industrial applications [7]. Lipase-producing microorganisms can be isolated from diverse habitats of decay materials like food materials, dairy industries, oil processing units, industrial wastes dumb yards, at ambient temperatures [8-9]. This report details the results of isolation, purification and characterization of one such highly active lipase producing fungal species from a soil collected in the nearby area of a petroleum crude oil processing plant.

MATERIALS AND METHODOLOGY

Sample Collection

Few grams of contaminated soil samples were taken in around a petroleum crude processing plant, transported in a sterilized container, and stored in a dark place at ambient temperature. The soil samples are mixed well before using them.

Isolation of fungal strains

Potato Dextrose medium containing 200g of potato, 20g of dextrose, 20g of agar and 1000ml of Distilled H_2O and adjusted to pH 6.6-7.2 was sterilized and cooled. Antibiotic-Ampicillin was added ascetically to the sterilized medium to avoid bacterial growth and poured into petri plates under aseptic conditions. 1.0g of soil sample was sprinkled aseptically over the medium, closed and the plates were incubated at 37°C for 2-4 days [10]. Serial dilution of a loop of each fungal colony observed in the agar plate was prepared. PDA agar plates of the serial dilution were incubated at 37°C for 2 days and checked for growth. Repeated sub-culturing by streaking on PDA medium was used to purify the culture. The isolates were maintained on PDA slants at 4°C.

Morphological identification of fungal isolates

The morphological nature, such as texture and color of the colonies was was observed. Fungal nature of the colonies was confirmed by lacto phenol staining. A loop of inoculums was mixed with a drop of distilled water on a clean glass slide. The culture was smeared and heat fixed. A drop of 95% ethanol was added to the smear and allowed to evaporate and then a drop of lacto phenol cotton blue stain was added. After 2mins the stain was washed away with distilled water and air dried. The smear was covered with a clean cover glass and the stained slides were observed under a 40x objective lens of a binocular light microscope (Labomed, India). The isolates were identified based on the shape of conidia and arrangement of spores on the mycelia [11].

Screening of lipase producing organisms using agar plates

The organism utilizes the carbon source of oil or ester present in the medium and grows uniformly around the inoculums well. Lipolytic organisms release lipase enzyme for the decomposition of the carbon source. The carbon source utilization is shown as a halo circular region of different color or structure. The diameter of the halo region can be taken as an indication, either of the ability of the enzyme to produce lipase enzyme or as the ability of the hydrolytic activity of the lipase enzyme [12]. Three agar plates containing olive oil and tributyrin substrates were used to confirm the relative lipase activity of strains as recommended by Jensen [13] and Smith & Hass [14]. They are-

1. Tween 80: 1g of tween 80 ester in 100ml of distilled water is mixed with 1.0g of peptone, 0.5g of NaCl, 0.01g of $CaCl_2.5H_2O$, 7g of $MgSO_4.7H_2O$, 7g of KH_2PO_4, 1.5g of agar and sterilized to prepare the medium and poured into Petri plate after sufficient cooling. Well was made in the solidified agar plate. After solidification wells were made in the agar. Stock solutions containing equal number of fungi of the different stains were added to the separate wells and allowed to incubate at 37°Cfor 48 hours. The diameter of the circular clear region around each well was noted. Larger the radius, higher is the lipase activity.
2. Rhodamine B: A sterilized medium containing 0.001g of Rhodamine - B, 0.8g of nutrient broth, 0.4g NaCl, 2g of olive oil and 1g agar in 100ml distilled water adjusted to pH 6.5 is prepared and poured into Petri plates. The stock culture with same concentrations was added to the wells and incubated. Formation of an orange fluorescent zone around the fungal colonies visible upon UV irradiation (350 nm) was noted and the halo diameter measured.
3. Phenol red: Agar plates containing sterilized medium components 0.01g Phenol red, 0.1g of $CaCl_2.5H_2O$, 2g of olive oil and 2g agar in 100ml 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 [15]

The purified fungal strain was identified by molecular tool methodology at CHROMOUS BIOTECH PVT.LTD, Bangalore. The analytical protocol consists of:

1. Isolation of the fungal genome DNA.
2. Amplification of the ITS region of the DNA with a high fidelity PCR Polymerase.
3. Sequencing of the PCR amplicons and
4. Building a phylogenetic tree.

The mycelium from the pure culture plates were pestle and mortared and treated with lysis buffer to release the genomic DNA. The fungal genomic DNA is extracted using a fungal genome isolation kit –RKT13.PCR amplification, was carried out with: Genomic DNA - 1 µl (100 ng), Forward Primer - 400ng, Reverse Primer - 400ng, dNTPs -(2.5mM each) 4 µl, 10X Taq DNA Polymerase Assay Buffer - 10 µl, Taq DNA Polymerase Enzyme - (3U/ µl) 1 µl, and water X µl to make up the total reaction volume as 100 µl. PCR amplification of the genomic DNA was carried out with an initial denaturation at 94°C for 5 min, followed by 35cycles of denaturation (at 94°C for 30 sec) and annealing (at 55°C for 30 secs), extension (at 72° for 60 sec), and a final extension (at 72°C for 7 min) at a final maximum Mg Cl_2 concentration of 1.5mM. The PCR amplified product was electrophorized in 1.2% agarose gel (with ethidium bromide) to know the bp size. Sequencing of the PCR amplified product was performed on ABI 3500xL 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. Sequencing was done by denaturation at 96°C for 1 min, followed by 25 cycles of denaturation (at 96°C for 10 sec), hybridization (at 50°C for 5 sec) and elongation (at 60°C for 4 min). The resultant nucleotide amplicons was analyzed with Seq Scape version 5.2 software, using BDTv3-KB-Denovo_v 5.2 protocol. Weighbor with alphabet size 4 and length size 1000,

utilizing the sequences aligned with a system software aligner Seq Scape_v5.2 was used to build the phylogenetic tree. Jukes-Cantor corrected distance model was used to generate a distance matrix.

RESULTS AND DISCUSSION

Potassium dextrose agar sprinkle plate of the petroleum contaminated soil showed three differently colored fungal colonies (Fig1).

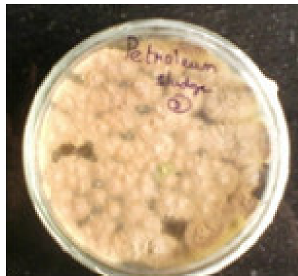

Fig 1 PDA Sprinkle Plate of Petroleum contaminated soil

A loop of each colored colony was serially diluted for purification. PDA pour plate of one such brown colony by serial dilution is shown in the Fig.2.

Figure 2a. Dilution 10^{-2} Figure 2b. Dilution 10^{-3} Figure 2c. Dilution 10^{-4}

FIGURE 2
Serial dilution Pour plates (2a,2b,2c) of brown coloured strain

Pure cultures plates of the different coloured stains isolated from the petroleum contaminated soil sprinkle plates are shown in Fig.3

Figure 3
Cultured plates of the three colored (brown, green and black) strains isolated from the petroleum mixed soil.

Microscopic analysis showed the presence of hyphae confirming the micro organism 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. Organisms that produce extra cellular lipases can hydrolyze the glycerides. The hydrolysis/ formation of the halo regions is a measure of their lipase activity. Tween 80 (Fig 4a, b, c) showed regions of hydrolysis for all the three colored colonies.

Figure 4a
Brown strain Fig 4b. Green Strain Fig 4c. Black strain
Lipolytic activity of the three strains on tween-80 agar plates. Fig 4a.of Brown strain.

This article can be downloaded from www.ijpbs.net

Figure 5a. Brown strain Figure 5b.Green Strain   figure5c. Black Strain

Figure 5
Lipolytic activity of the three strains on Rhodamine-B containing plates.
Fig 5a.of Brown strain.Fig 5b.of Green Strain and Fig 5c.of Black strain

Rhodamine-B (Fig 5a and 5b) of green and black colored colonies only showed hydrolysis of olive oil while the brown colored colony was not showing any region of lipase activity. Phenol Red agar plates did not show any pattern for all three colonies. The green and black colonies were showing lipase activity on both fatty ester and olive oil. Among them black colored strain exhibited larger diameter of halo region indicating higher lipase activity among all the three colonies. Black colored strain was hence taken for identification and further optimization studies. The agarose gel analysis of the extracted genomic DNA is shown in Fig.6.

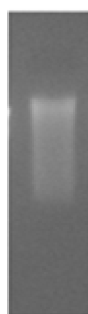

Figure 6
Electrophorized agarose gel containing the extracted genome of the black fungal strain

Agarose gel analysis, showed the PCR amplified product of the ITS region of the fungal genome to be of 600bp length as in Fig.6.

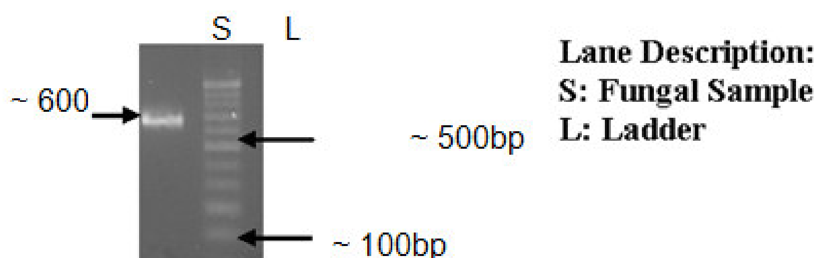

Figure 7
Electrophorosis agarose gel analysis of the ITS region of the genome The 600bp nucleotide sequence of the ITS region of the black colored fungi is given below in fig 8

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1   CTTCCTGTAG GGTTAACCTG CGGAAGGATC ATTACCGAGT GCGGGTCCTT   50
51  TGGGCCCAAC CTCCCATCCG TGTCTATTAT ACCCTGTTGC TTCGGCGGGC  100
101 CCGCCGCTTG TCGGCCGCCG GGGGGGCGCC TTTGCCCCCC GGGCCCGTGC  150
151 CCGCCGGAGA CCCCAACACG AACACTGTCT GAAAGCGTGC AGTCTGAGTT  200
201 GATTGAATGC AATCAGTTAA AACTTTCAAC AATGGATCTC TTGGTTCCGG  250
251 CATCGATGAA GAACGCAGCG AAATGCGATA ACTAATGTGA ATTGCAGAAT  300
301 TCAGTGAATC ATCGAGTCTT TGAACGCACA TTGCGCCCCC TGGTATTCCG  350
351 GGGGGCATGC CTGTCCGAGC GTCATTGCTG CCCTCAAGCC CGGCTTGTGT  400
401 GTTGGGTCGC CGTCCCCCTC TCCGGGGGGA CGGGCCCGAA AGGCAGCGGC  450
451 GGCACCGCGT CCGATCCTCG AGCGTATGGG GCTTTGTCAC ATGCTCTGTA  500
501 GGATTGGCCG GCGCCTGCCG ACGTTTTCCA ACCATTTTTT CCAGCCCCGA  550
551 CCTCGGATCA GGTAGGGATA CCCGCTGAAC TTAAGCATAT CAATAAGGCG  600
601 GAG
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Figure8
600bp nucleotide sequence of the ITS region of the Black Fungi

The Phylogenetic tree analysis of the black strain is shown in Fig 9.

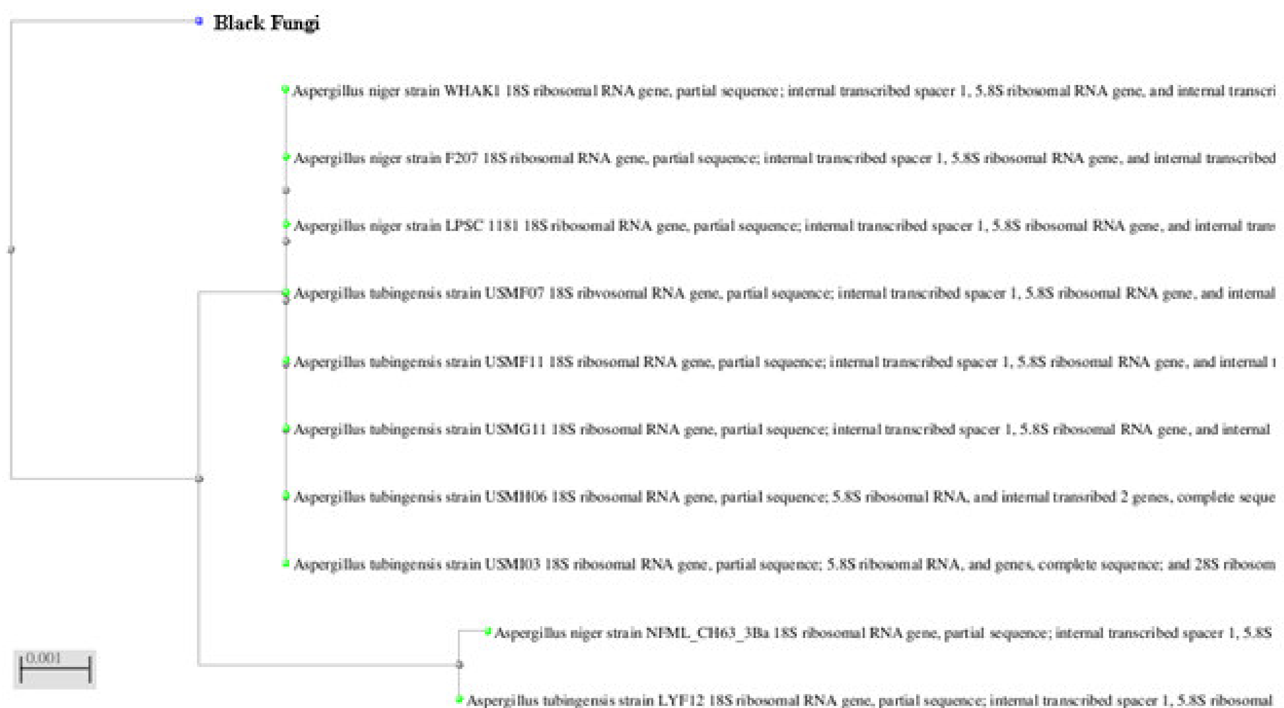

Figure 9
Phylogenetic tree relationship of the black fungi.

The Fungal sample was found to be *Aspergillus niger* strain with close resemblance to WHAK1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (NCBI Acc No: Sequence ID: gb|JQ929761.1) The next closest homologue was found to be *Aspergillus niger* strain NFML_CH63_3Ba 18S ribosomal RNA

gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Sequence ID: gb|KM458840.1

CONCLUSION

We are interested in the identification of extra cellular lipase producing fungal strains, for converting vegetable oils into alcohol esters to be used as bio diesel. Three fungal strains were isolated from the soil spilled with petroleum stock of a petroleum refinery. The relative hydrolytic activities of their lipase enzymes were tested. A black colored strain showing relatively higher hydrolytic activity was characterized to be *Aspergillus niger*. The species is available with accession number KR261667.

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