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RESEARCH ARTICLE

Isolation, screening and identification of Lipase producing fungi from cotton seed soapstock

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Abstract

Background/Objectives: The present study was focused to exploit the indigenous strains of fungi isolated from cotton seed soapstock for the production of the extracellular lipase through submerged fermentation technique. **Methods/ Statistical analysis:** Cotton seed soapstock samples used in the study contains gelatinous oil richer chemical constituents. In addition, their enrichment and diluted materials were used for the isolation of lipase producing microorganisms on tributyrin agar plates. All isolates were lipase positive confirmed by a qualitative plate assay. Quantitative estimation of Lipase production activity was measured spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as a substrate. In order to exploit the isolated fungal strain for industrial usage, other cellulase and protease enzymes were tested by plate assay. Morphological and molecular characteristics of selected isolates were studied. **Findings:** From enriched soapstock samples, a total of 49 cultures were isolated among them 19 fungal isolates were screened for lipase, cellulase, and protease activity qualitatively by plate assay. Out of 19, six fungi were selected based on their lipase activity. Highly potent *Fusarium solani* 7F had the ability to produce 5.95 U/mL/min. crude lipase whereas *Aspergillus niger* 13F has 4.2 U/mL/min. after 4days incubation at 30^{o}C. Potent fungi culture was identified by morphological, cultural, and molecular characteristics (18s rRNA gene sequence and phylogenetic analysis) revealed them as *Penicillium griseofulvum* 5F, *Aspergillus flavus* 6F, *Fusarium solani* 7F, *Aspergillus niger* 12F, *Aspergillus niger* 13F, and *Aspergillus terreus* 17F. **Novelty:** Fungi was the first time reported and isolated from cotton seed soapstock materials. In future studies, this enzyme will be used in the degradation of soapstock and also in the production of biosurfactant from soapstock.

Keywords: Cotton seed soapstock; Fusarium solani 7F; p-nitrophenyl palmitate; Tributyrin agar plates; degradation

1 Introduction

Lipases (triacylglycerol ester hydrolases E.C.3.1.1.3.) are one of the most important industrial enzymes catalyze both the hydrolysis and the synthesis of esters from

glycerol and long-chain fatty acids. These enzymes, under specific condition to catalyze reversible reactions: interesterification, aminolysis and transesterification reactions [1,2]. Novel enzymes research of lipase may provide greater understanding of previously discovered enzymes and their functional significance using molecular tools that may be used as parts of the microbial pool for production of lipase in research at laboratory and industries level [3]. Research aspects, reported lipase producing fungi were *Humicolal anuginosa* [4], *Fusarium* sp. [5–7], *Mucor* sp. [8], *Aspergillus* sp. [9,10] *Rhizopus oryzae* [6,11], *Colletotrichum gloeosporioides* [12], *Alternaria dianthicola*, *Curvularia* sp., *Penicillium* sp., *Trichoderma viridae*, *Macrophomina phaseolina* [6], *Hypocrea pseudokoningii* [13] etc. Lipases are a ubiquitous enzyme that is found widely in a variety of natural sources industrial wastes, vegetables oil processing factories, soil contamination with oil, etc [14]. Lipid is a large constituent of the earth's biomass and has its application in various industries like in detergents, dairy and textile, production of surfactants, oil processing and biodiesel of microbial origin [15,16].

Cotton is a source of fiber, oil and protein. Oil-rich seeds are the vigor of pathogenic fungi in the process of biodeterioration of seed may be related to their degree of lipase production [6]. Soapstock is a gelatinous dark brown undesirable chemical compound which is separated from the oil refinery [17]. Soapstock will account for higher values for 5 to 10% of the crude oil mass and high concentration of free fatty acids and these byproducts use for fungal lipase production in SSF *Aspergillus niger* [18]. Soapstock from alkali refining is a source of fatty acids, but it also presents a handling, storage, and disposal problem. It is generated at a rate of ~6% of the volume of crude soybean oil caustic refined [19].

The production process, oils from agro-industrial waste can come forward as efficient enzymes inductor reducing the production cost. Additionally, this process can reduce environmental problems related to agro-industrial disposal [20]. Screening for new microorganisms and their lipolytic enzyme will open simple routes for synthetic processes and consequently new and faster ways to solve environmental problems. In this regard, the biotechnological process that can added value to waste by biodiesel production via lipase enzyme esterification [21,22]. On other hands microbial treatment to soapstock, *Staphylococcus* sp. strain produced biosurfactants and extracellular lipase, when soapstock was used as an alternative carbon source. These potent metabolic product biosurfactant and lipolytic enzymes were potentially applicable in soap stock treatment [23]. *Pseudomonas aeruginosa* strain was utilized Soybean oil soapstock as the sole carbon source for the production of rhamnolipids [24]. *Candida antarctica* and *Candida apicola* were produced glycolipids when supplemented with soapstock [25]. *Oospora lactis* fungi utilized cotton oil soapstock as carbon source and produced lipase enzyme. During fungi cultivation on soapstock, enzymatically hydrolysis of fat was taken place on the approach of biological alteration of cotton oil soap stock [26]. Lipase producing fungal strains, *Aspergillus*, *Penicillium*, *Trichoderma* and *Mucor* were isolated from palm oil mill effluent composts [27]. Where Lipase producing *Bacillus licheniformis* and *Bacillus pumilus* were isolated from cotton soapstock which will used in enzymatic degradation of soapstock [28]. Lipase produced by *F. solani* isolated from leaves decomposed in an aquatic environment has great potential to application in biodiesel production by transesterifcation of vegetable oils, as well as food industries in the production of fatty acid esters by hydrolysis and esterifcation. In the present study, maximum lipase activity was achieved by *Fusarium solani* using cotton oil as substrate and there is no any work related to isolation of lipolytic fungi by using cotton soapstock as a natural source.

This study was conducted to isolate lipase producing fungi that were enriched and screened on tributyrin agar plates. Culture was further analyzed for cellulase and protease production by plate assay. The fungal strain was identified on the basis of morphological characteristics and molecular 18s RNA sequence (Genetic Characterization) of the cultures using manual partial gene sequencing. In future study lipase will used in degradation of soapstock and produce industrially important products.

2 Materials and Methods

2.1 Sample collection

Lipolytic fungal strains were isolated from cotton seed Soapstock samples collected from different cotton seed oil refinery industries situated at Kadi (North Gujarat), India. All the soapstock samples were collected at the site of Washer discharge end of the pipe in sterile containers.

2.2 Enrichment process of Soapstock sample

For enrichment B/H (Bushnell–Haas) medium was used [29–31]. 10 grams of Cotton seed oil soapstock samples were enriched in 100 ml of B/H mediums for the growth of microbes. Broths were incubated at 30^{o}C in static condition for 5 days. From each enriched samples, 1ml of samples were inoculated to 100 ml of the Trybutyrine broth medium. TBA broth was incubated at 30^{o}C, in static condition for 48 hours. Enrichment was performed over 7 days of incubation.

2.3 Isolation of Lipolytic Fungi

For isolation of fungi, A series of dilutions from 10^{-1} to 10^{-10} were prepared from enriched sample. These dilutions were used in spread plate method. The diluted samples (100 μl) were pipette and spread on Tributyrin agar plate and incubated at 30^{o}C for 7 days. Isolated fungal colonies were sub-cultured and purified on the TBA medium. Isolated cultures were preserved at 4^{o}C temperatures in Potato dextrose agar slants for further works.

2.4 Qualitative screening of Lipase producing fungi

For screening, Qualitative plate assay was performed according to the method reported earlier for lipase-producing strains selection [31]. Isolates plugs were inoculated on tributyrin agar plates and incubated at 30°C for 5 days. The clear zone was observed due to the hydrolysis of tributyrin by lipase.

2.5 Lipase enzyme assay

2.5.1 The composition of production medium for Lipase enzyme

In 250 ml Conical flask 100 ml of production medium [31] were prepared for fungal culture. Production medium for lipase contain 0.5% Peptone, 0.5% Yeast extract, 0.5% NaCl, 1% Cottonseed Oil, pH-7.

2.5.2 Quantitative analysis of Lipase enzyme under submerge fermentation

Fungal cultures that showed Positive lipase production in plate assay were subjected to quantitative analysis. 5 days old cultures grown on the TBA medium were used for inoculation. Two plugs of fungal culture were inoculated into 100 ml of production medium for lipase production. Cultures were incubated at 30°C and 100 rpm for 5 days. The enzyme assay was performed according to the method reported by Patel and Shah, 2018 [32]. The culture filtrate was removed from each flask at every 24 hrs interval and centrifuged at 10,000 rpm for 10 min at 40°C. Supernatant were used for enzyme assay. By using p-nitro phenyl palmitate (p-NPP) as a substrate, lipase production was determined by a spectrophotometric assay. Lipase hydrolyzed p-NPP to give p-NP which gave yellow color and absorbance of which was measured spectrophotometrically at 410 nm against enzyme free blank.

2.5.3 Assay of enzyme

The substrate solution preparation: solution A (40 mg of p-NPP dissolved in 12 ml isopropanol) and solution B (0.1 g of gum Acasia and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water). The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring to obtain an emulsion that remains stable for 2 h. The assay mixture contains 1 ml of the substrate, 0.5 ml of buffer (Potassium phosphate buffer, pH 7, 0.1 M) and 1 ml of the enzyme. The volume was made up to 4 ml with distilled water. Incubate at RT for 5 min. Lipase activity was stopped by adding 1 ml 0.2M NaOH. Lipase activity was measured by monitoring the hydrolysis of 5mM p-nitro phenyl palmitate (pNPP) buffered with 0.1 M Potassium Phosphate buffer (pH 7.0) at 410 nm for 5 min. One unit of lipase enzyme activity defined as 1mM of p-NP liberated per min under the assay conditions.

2.6 Enzyme profile

Qualitative screening for Cellulase and Protease were done in nutrient agar media containing 1% carboxy methyl cellulose and 1% skim milk substrates respectively. Culture plug was inoculated and incubated at 30°C for 5 days. Zone of clearance was observed around the colonies due to the utilization of the particular substrate.

2.6.1 For Cellulolytic activity

As per method performed earlier [3], culture were grown in medium containing 1% carboxy methyl cellulose. After incubation 0.1 % congo red staining solution was added in CMC plates, discard stain after 5 min and the plates were destained by 1M NaCl solution with continuous stirring for 15-20 min. The clear zone around colonies indicated cellulose hydrolysis.

2.6.2 For Proteolytic activity

1% skim milk substrate containing nutrient agar media were used for screening of protease [33]. Culture plugs was inoculated and incubated at 30°C for 5 days and observed for clear zone around colonies due to protein hydrolysis.

2.7 Identification of the fungal isolates: morphological and molecular characteristics

The potent isolates showing the maximum zone of clearance were selected for further analysis. On potato dextrose agar medium, morphological and cultural characteristics of the isolates were studied such as the color of hyphae, type of hyphae, shape and the characteristics of spore, using microscopic examination. Molecular characterization of potent fungal strains was done by 18s rRNA partial gene sequencing analysis. It was carried out at the Biogene department of Gujarat State Biotechnology Mission (GSBTM), Gandhinagar. Fungi culture isolates were identified through partial gene ITS region sequence and 18S rRNA sequence using ITS 1/ITS 4 primer. The 18S rRNA gene sequence was used to carry out BLAST with the nr database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using the RDP database and the phylogenetic tree was constructed using MEGA X.

2.8 Statistical analysis

In all experiments, three sets of reading were taken. Data, where appropriate, were subjected to two- way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and student's test using Graph Pad Prism 8.3.1 (GraphPad Software Inc., San Diego, CA, USA). Quantitative analysis of lipase production were plotted using Graph-Pad Prism 8.3.1, Significance was accepted at $p<0.05$.

3 Results and discussion

3.1 Sample collection

Samples were collected from two oil refinery industries, Uma oil refinery industry, Kadi (North Gujarat), India & Mahakali oil refinery industry, Karannagar (North Gujarat), India.

3.2 Qualitative screening and isolation of lipolytic fungi

A total of 49 pure cultures were isolated from enriched soapstock samples. Among these, 19 cultures were fungal isolates. The fungal cultures were further subjected to the qualitative screening on tributyrin agar medium to select lipolytic strain. The clear zone around fungal isolates exhibited on tributyrine agar plates within 5 days indicates positive results. Range of 5.30 to 35.00 mm of clear zone of lipid hydrolysis was observed by lipase producing isolates. However, only six isolates that showed good zone of lipid hydrolysis in diameter ranged between 13.30 to 35.00 mm were selected for further study and results were represented (Table 1, Figure 1). Among the six fungal strains, 7F isolate was detected to produce maximum zone of hydrolysis (35.00 mm), whereas 12F isolate was produced minimum zone hydrolysis (13.30 mm) on 1% tributirine agar plates.

Table 1. Qualitative enzyme activity measure in clear zone diameter to colony diameter ratio of fungal isolates.

Sr.no	Fungal Isolates	Clear zone diameter ratio (mm)		
		Lipase activity	Cellulase Activity	Protease Activity
1	5F	16.00 mm	10.34 mm	08.68 mm
2	6F	19.70 mm	13.29 mm	11.90 mm
3	7F	35.00 mm	29.10 mm	15.00 mm
4	12F	13.30 mm	09.00 mm	07.39 mm
5	13F	20.00 mm	17.90 mm	14.17 mm
6	17F	16.30 mm	13.37 mm	10.00 mm

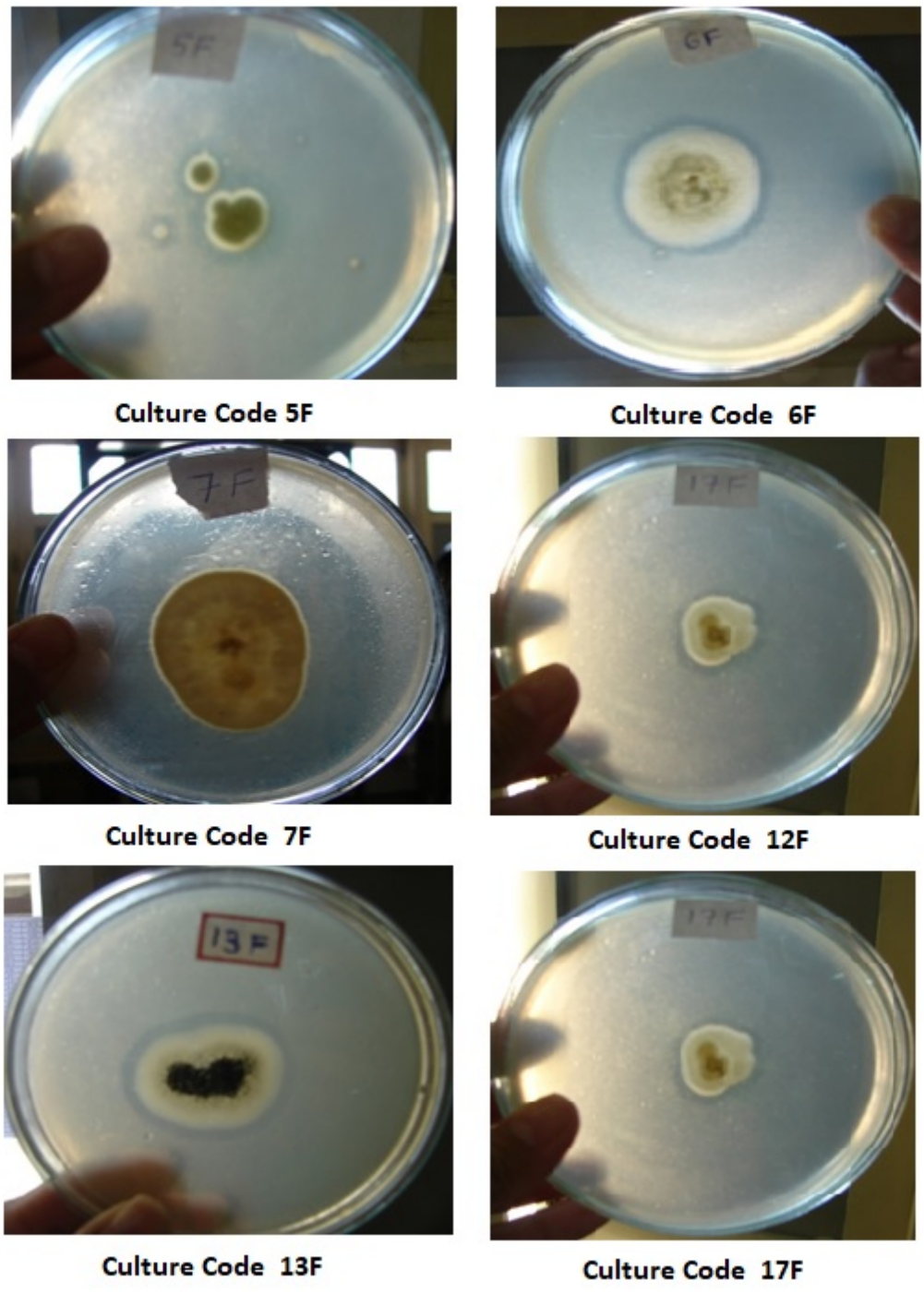

Fig 1. Qualitative screening of Lipase production by fungal cultures on 1% tributyrin agar plate

3.3 Quantitative analysis of Lipase enzyme under submerges fermentation

Agro-industrial wastes can easily be degraded by filamentous fungi, when synthesizing industrially important bio-compounds, such as lipolytic enzymes [34]. It is known that various fats, fatty acids, plant oils, triglycerides, ester-based detergents, and other substances are the best inducers of lipase synthesis by microorganisms and sources of carbon [35]. From earlier reported result *Aspergillus* species were used oils from agro-industrial waste (grape seed oil (GSO) and cotton seed oil (CSO)) to enhance lipase production further higher percentage of fatty acid esters (>80%), namely, soybean, olive, and GSO, promoted the highest lipase production [20]. Present study results supports the earlier report on use of cotton seed oil as carbon source to enhance lipase production by *Penicillium melinii* [35] and *Oospora lactis* fungi utilized cotton oil soapstock (oil richer) as carbon source and produced lipase enzyme [26]. *Fusarium* sp. are able to cause disease in plant growth stages [36,37]. In contrast they are good industrially important lipase producers. Growth of fungi was enhanced in the production medium supplemented with oil. High fungus mycelium, biomass and maximum crude lipase production were obtained on 4th day in submerged fermentation are comparable with the previous studies reported results on *Aspergillus* sp. strains [9]. The lipase producing strains *Aspergillus* spp. were more frequently present in palm oil mill effluent composts and were isolated from it [27].

Lipolytic strains were further quantitatively tested for crude lipase production by p- NPP as a substrate. In plate assay, short-listed six fungal isolates were further screen for lipase production by submerged fermentation. The enzyme activities were performed by all six selected isolates. The six selected fungal isolates (5F, 6F, 7F, 12F, 13F and 17F) were examined for lipase production after 120hrs cultivation in 100 mL liquid medium supplemented with 1% cotton seed oil as a substrate. In previously reported *F. solani* was isolated from decomposition of leaves in an aquatic environment, shown lipase production when utilizing Cotton oil as a carbon source in submerged condition [28]. Similar result was obtained; *Fusarium solani* 7F fungi produced lipase in presence of cotton seed oil in submerged fermentation. The activities ranged from 2.1 to 5.9 U/mL/min for lipase. From the observation, the 7F isolate exhibited the highest lipase activities, 5.9 U/mL/min than that the others. The lipase activities were shown by individual fungal isolates at different time period (Figure 2). 7F fungi showed maximum lipase activity 5.95 U/ml/min in submerged fermentation when incubated at 30^0C at pH 7. The activity of 7F, when compared to other fungi was highly significant ($p<0.0001$) at a time interval of 24hrs. Lipase activity (5.92 U/mL/min) of 7F was obtained maximum compare to lipase activity (5.72 U/mL/min) of *Mucor* [27].

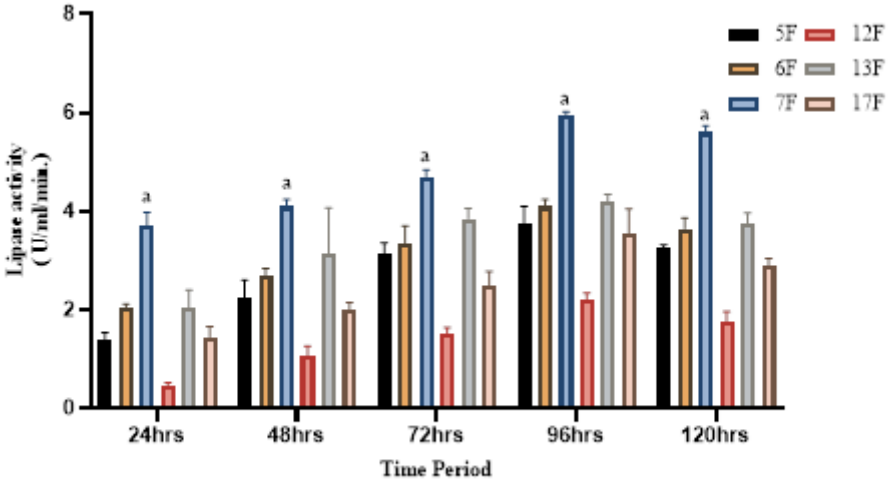

Fig 2. Time course study of different fungal isolates. ANOVA: p value = $p < 0.0001$, p value summary based on Tukey's multiple comparison tests = a highly significant at a time interval of 24hrs.

3.4 Enzyme profile

Fungi cultures were further screened for Cellulase and Protease activity by plate assay. Their cellulase and protease activity were detected by clear zone formation on CMC and milk agar plate respectively within 5 days and results were represented (Table 1). All six isolates showed positive result for cellulase and protease activity in plate assay. Among the six fungal strains, 7F isolate

was detected to produce maximum zone of hydrolysis (29.10 mm) on 1% carboxy- methyl cellulose agar plate, whereas 7F isolate was also detected to produce maximum zone hydrolysis (15.00 mm) on 1% skim milk agar plate.

3.5 Characterization of Fungal Isolates

The fungal isolates which showed the maximum zone of clearance for lipase production were subjected to further characterization and identification. The morphological and cultural characteristics of *Fusarium solani* were the same as those reported in previous work [36]. Growth characteristics of the *Fusarium solani* 7F isolate on PDA plate were white mycelia, which spread very fast, hyphae branched and septate. *Fusarium solani* 7F grows radials and spores produced. Yellowish pigments appear on the back of the plate. *Penicillium griseofulvum* 5F fungi isolate grow slowly, mycelia green cottony and back of plate yellow pigment. *Aspergillus flavus* 6F culture mycelium spread fast, mycelia white initially after three days incubation macro spore turn green and back of plate no pigment. Zone observed in two parts white to green centre. *Aspergillus niger* 12F fungus moderately grows on PDA, mycelia white cottony and no pigment observed. *Aspergillus niger* 13F isolate growth very fast, mycelia white initially on aging macro spore turn black and back of plate no pigment. Zone observed in two parts white to black centre. *Aspergillus terreus* 17F culture growth was fast, mycelia initially white after three days incubation macro spore turns brown. Zone observed in two parts white to brown centre. Potent fungi culture was identified by morphological, cultural and molecular characteristics culture 5F, 6F, 7F, 12F, 13F and 17F were identified as *Penicillium griseofulvum*, *Aspergillus flavus*, *Fusarium solani*, *Aspergillus niger*, *Aspergillus niger* and *Aspergillus terreus* respectively (Figure 3). The sequence of the gene deposited at the NCBI repository is accessible by Accession numbers (Table 2). The Phylogenetic tree was created with maximum likelihood methods and Tamura-Nei model shows evolutionary history of deposited sequence with other aligned sequence. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated Taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA X (Figure 4). Reported fungal strains such as *Aspergillus flavus*, Aspergillus niger and *Aspergillus terreus* showed the degradation of crude oil due to the production of extracellular enzymes [38].

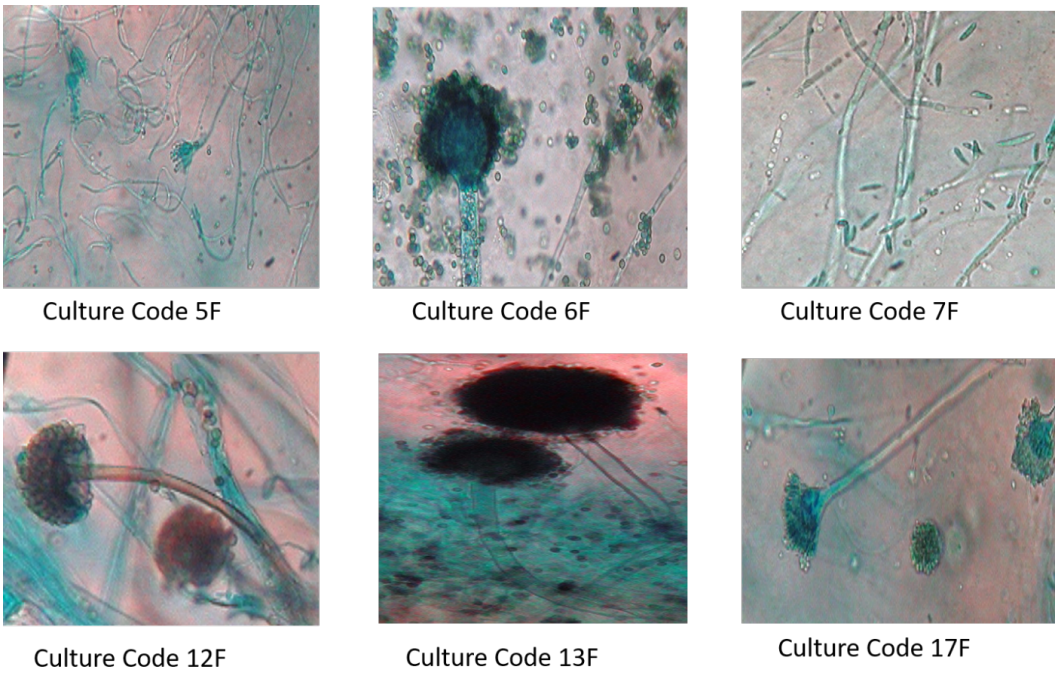

Fig 3. Microscopic observation of isolated fungi

Table 2. Accession number of isolated Fungi

Sr.no	Culture code	Accession number	Organism
1	5F	MN749934	*Penicillium griseofulvum*
2	6F	MN750585	*Aspergillus flavus*
3	7F	MH571778	*Fusarium solani*
4	12F	MN750583	*Aspergillus niger*
5	13F	MH571779	*Aspergillus niger*
6	17F	MN750586	*Aspergillus terreus*

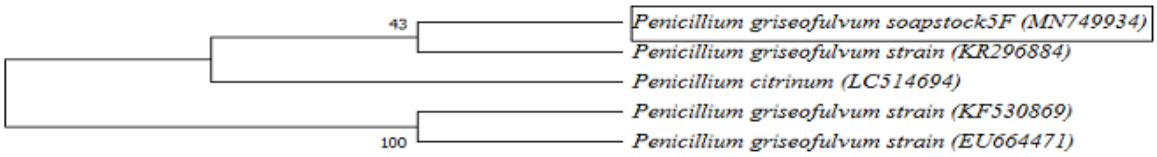

Figure 4a. Phylogenetic tree of *Penicillium griseofulvum soapstock5F (MN749934)*

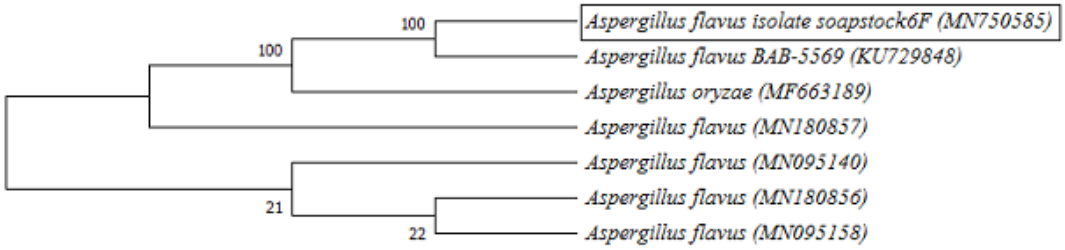

Figure 4b. Phylogenetic tree of *Aspergillus flavus soapstock 6F (MN750585)*

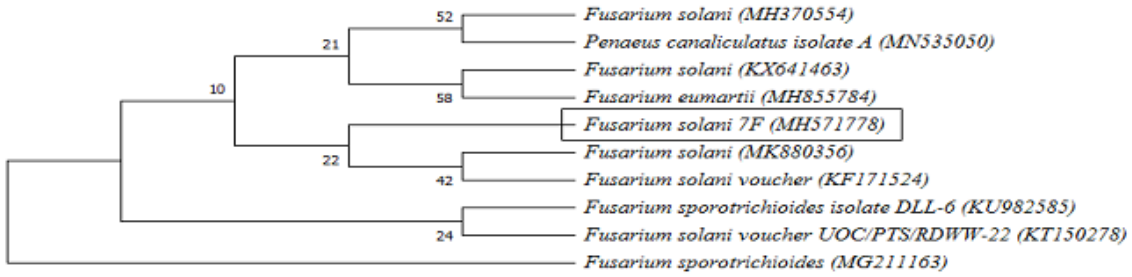

Figure 4c. Phylogenetic tree of *Fusarium solani 7F (MH571778)*

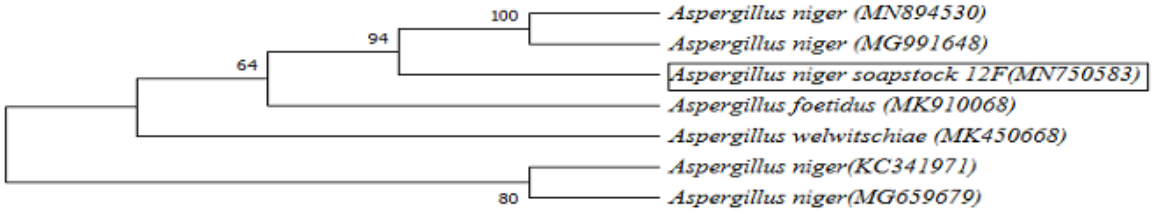

Figure 4d. Phylogenetic tree of *Aspergillus niger soapstock 12F (MN750583)*

Fig 4.

4 Conclusion

Cotton seed soapstock samples were collected from the oil refinery industry for the isolation & screening extracellular lipase producing micro-organisms. 49 isolates including bacteria & fungi were screened. The isolates showed highest lipase production in plate assays were further quantitatively tested for production of lipase by pNPP as substrate assay. The isolated micro-organisms have higher ability to produce lipase enzyme. In further studies pilot scale lipase production and its purification studies will be conducted. Enzyme derived from *Fusarium solani* 7F is further used in the microbial degradation of soapstock and production of biosurfactant. The identified cultures were deposited in the NCBI culture collection center with accession number.

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