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Isolation, Screening and Optimization of Lipase Producing Fungal Strains from Agricultural Soil

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Abstract

Potent fungal lipase producers have been isolated from paddy field of agricultural soil was studied in submerged batch fermentation. Total 14 fungal isolates were obtained by using serial dilution technique. Preliminary qualitative screening was done by using Tributyrin agar medium for the secretion of lipase from fungal isolates. Six fungal isolates out of the 14 isolates were shown clear holo zone in TBA plates after 3-7 days of incubation. Quantitative screening was done for the production lipase; shake-flask culture method has been used. In present investigation for the modification of culture condition and nutrient source it have been optimized by involving various parameters such as pH, time course, inoculum volume, agitation rate as well as various substrates were evaluated in this study. Lipase was detected in culture filtrate by using filtration technique. From the results, revealed that the isolated fungal cultures are alkaline in nature as because of the medium pH of 8.0 and temperature range of 35°C when incubated up to 96 hours in 3 ml of inoculum volume and 150 rpm agitation rate were optimum for maximizing lipase production under submerged fermentation by fungal isolates i.e. mostly of belongs to genera *Aspergillus* sp. Under Submerged fermentation the mineral growth medium (MGM) contained (in g/L) NaH_2PO_4: 12.0, KH_2PO_4: 2.0, $MgSO_4.7H_2O$: 0.3 and $CaCl_2$: 0.25. Ammonium Sulphate at 1% and mustard oil - 2% were used as carbon and nitrogen source respectively. Highest Lipase activity were obtained from CAK7 (*Aspergillus tamarii*) and CAK9 (*Aspergillus niger*). Maximum lipase activity 88.74 U/ml obtained by *Aspergillus niger* sp. followed a logarithmic pattern on the 4th day of fermentation. By using Statistical approach (OFAT) revealed that the culture conditions significantly ($p<0.05$) influenced lipase production by these fungal isolates. In this investigation results obtained for the production of extracellular lipase under submerged fermentation mustard oil could be used as a substrate inducer would be economically cost effective and beneficial for industrial applications.

Keywords
Lipase, Agricultural Soil, *Aspergillus* sp., Optimization Parameters, Submerged fermentation

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INTRODUCTION:

Lipases are ubiquitous in nature, generally lipase enzymes are produced by animals, plants and microorganisms. They are catalyzing the hydrolysis of fats / oils into fatty acids and glycerol at the water–lipid interface and reverse reaction obtained in non-aqueous media. Lipases [triacyl glycerol acylhydrolases (E.C.3.1.1.3)] have been secured the prominent place among all the biocatalysts due to their novel and multi-fold applications in oleochemistry, organic synthesis, detergent formulation and nutrition [17]. Microbial lipase producers are most significant for their special industrial attention as because of their stability, selectivity and broad substrate specificity. Numerous microorganisms are produces extracellular lipases, including bacteria, yeast, few protozoa and fungi for the digestion of lipid materials [29]. Fungi have been recognized as a best lipase source among the various microorganisms [3]. Fungal lipases play an important role in developing various industrial products. Fungal lipases have high substrate specificity, which make it very attractive for industrial applications. Lipases can be more beneficial for industrial enzymes are increasingly produced via recombinant DNA technology [27]. Fungal lipases are mostly used for the oil degradation, fatty acid, fatty acid alcohol or fatty acid ester and surfactants for induction. By using the fermentation process to enhance the activity of lipase since lower costs of production could promote new industrial applications. Production of lipase is affected by physio-chemical parameters such as temperature, pH, medium composition and presence of inducers among others. Many investigators have been studied for the factor affecting on extracellular lipase production.

Therefore, in the present investigation, a study was undertaken to isolate, screening lipase producing fungal strains from paddy field soil, and to optimize the physical parameters under submerged fermentation, to enhance the production of lipase it would be cost effective on basal substrate medium.

MATERIALS AND METHODS:

Isolation of lipase producing microorganisms:

A significant lipase producing strains isolated from agricultural soil of Paddy field, Achhoti Village, Kumhari, near Bhilai Region, Chhattisgarh. The samples were inoculated to the potato dextrose agar (PDA) followed by ascertaining the purity of cultures on the basis of cultural and morphological features [2, 11], was subculture on Potato dextrose agar media supplemented with streptomycin (20 µg L-1) at 28°C for 3-5 days and maintained at 4°C. Molecular identification was done by 18sRNA sequencing method and suitable phylogenetic tree has been constructed as per standard protocol of white [4] and it has been submitted to NCBI with the Accession number.

Preliminary selection of strains for lipase activity (Tributyrin agar plates):

Primary selection of lipase producing fungal strains was on the basis of diffusion zone of the enzyme (diameter, cm) secreted into the agar medium [28]. Preliminary lipolytic screening was carried by using medium composed of (in g · L-1): mycological peptone (5.0), yeast extract (3.0), agar (20.0), and Tributyrin (glycerine tributyrate) (Sigma-Aldrich) added to give a final concentration of 0.1 % (v/v), pH 6.0. After autoclaving the medium (121°C), the mixture was emulsified by shaking for 15 min and after cooling to approximately 60 ºC, the transferred the medium into petriplates and rapidly cooled [3]. The microorganisms were incubated at 30°C. Lipolytic activity was identified on the plates as a transparent halo around the colonies after 7 days of incubation [5]. Production of lipase indicated by the formation of clear halos around the colonies grown on agar plates containing tributyrin.

Lipase Production in Submerged Fermentation:

According to Falony [1] for lipase production under SmF, a mineral growth medium (g/l) containing, NaH_2PO_4 -12.0, KH_2PO_4 - 2.0, $MgSO_4$. $7H_2O$ - 0.3, $CaCl_2$ - 0.25, Ammonium Sulplate - 1% and olive oil - 2% were autoclaved at 15 psi for 20 minutes. Followed by sterilization, the flasks were inoculated with 1.0 ml in 0.1% Tween 80 (v/v) of spore suspension (80.6 × 10^4 spores/ml) taken from a week old slant and inoculated at 28°C for a week in shaker (Orbital Shaker, Remi @120rpm) with an initial pH 6.0 [6]. The contents were filtered through Whatman filter paper (No. 1).

Extraction of Crude Enzyme and Determination of Enzyme Activity:

After the 5^{th} day of incubation the spent broth was extracted by the filtration through Whatman filter paper (No. 1). The culture filtrate thus obtained by centrifugation at 8000rpm for 20 minutes leads to a clear supernatant to be used as crude enzyme [30].

Lipase activity was estimated by using p-nitrophenyl palmitate (pNPP) (Sigma, USA) as substrate. As per the standard protocol of Ertugrul[7] substrate solution was prepared by freshly mixing solution-A (30 mg of pNPP in 10 ml of isopropanol) with solution-B (0.1 gm of gum Arabic and 0.4 ml of Triton X-100 in 90 ml of 50 mM Tris-HCL buffer, pH 8.0)

while stirring until all were dissolved. The mixture of 9 ml of substrate solution and 1 ml of enzyme solution was incubated at 60±0.1°C for 15 min and the absorbance measured at 410 nm (UV/VIS spectrophotometer, Shimadzu). The coefficient of extinction (ε) of p-nitrophenol (pNP), under conditions described, was determined from the absorbance at λ = 410 nm of standard solutions of pNP (0.01 to 0.1 mmol/ml) (ε410 = 14.653 L/mol/cm). Suitable controls were made for each experiment. One unit of enzyme activity was expressed as 1 µmol of p-nitrophenol released per minute under the assay conditions [6]. Dissolved protein concentration was determined according to Bardford method, by using bovine serum albumin as a standard.

Optimization of Bioprocess variables for lipase Producing Fungal Strains:

Effect of Incubation Time on Enzyme Activity

To determine the maximum lipase activity were determined by incubating the inoculated media for the amount of maximum lipase produced was determined daily for a period of six days (12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 hrs) in the formulated medium with the best parameters for lipase secretion. Samples were analysed at 12 h intervals for enzyme activity as discussed earlier [6, 16].

Effect of Inoculum Volume on Lipase Activity

For maximal enzyme production was evaluated using different volume of conidial inoculum (1, 3 and 5ml) prepared. In basal medium the prepared spore suspension was inoculated and incubated at 35°C for 4 days. After the 4th day of incubation determine the enzyme activity by using Okpalla [12] method.

Effect of Different Substrate on Lipase Activity

Maximum enzyme activity was evaluated by using different oil substrates (2% conc. Selected). Six different oils, viz., olive oil, soybean oil, groundnut oil, coconut oil, mustard oil and tween-80 were used for the study. After 4 days of incubation, enzyme activity was estimated as discussed earlier [3].

Effect of Incubation Temperature on Lipase Activity

Optimum incubation temperature for maximum enzyme production was evaluated by incubating the inoculated media with mustard oil (selected as optimal substrate based on the previous analysis). According to the Francis [3] the following temperature 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C for 4 days of incubation, temperature was already selected by previous experiment and enzyme activity was determined as discussed earlier.

Effect of Initial pH of the Medium on Lipase Activity

Effect of different culture conditions such as pH of medium on the activity of lipase enzyme at initial pH determined by adjusting the pH of the medium to various levels i.e. 2, 4, 6, 8 and 10 with either 1.0 N HCl or 1.0 N NaOH (Vishwe V.S. *et al.,* 2015) for maximal enzyme activity was estimated after 4 days of incubation at 35°C by Francois [15].

Effect of Agitation on Lipase Activity

Effect of agitation on enzyme production was analysed by incubating the minimal media by taking in shake flasks conditions at different rpm (100, 150, 200 and 250 rpm) and enzyme assayed was analysed by using pNPP as substrate solution after 4 days of incubation at 35°C.

Statistical Approaches:

For the optimization of various parameters for the enhancement of lipase production was used statistical approach such as one factor at a time (OFAT).

Time Course Study under Optimal Condition:

Time course experiment was conducted with the optimized parameters which is determined after statistical optimization of various variables.

RESULTS AND DISCUSSIONS:

Lipase synthesizing potent strain:

In this study, initially 14 were labelled as CAK1 to CAK14 fungal isolates occurred from the paddy field of agricultural soil, most of fungal isolates belongs to the genera *Aspergillus sp.* by preliminary culture characterization, morphological and microscopic identification by lactophenol cotton blue staining technique. Qualitative estimation was done for the potent lipase producing fungal strain to the formation of clear halos around the colonies grown on agar plates containing Tributyrin agar plates. Six (CAK3, CAK5, CAK7, CAK8, CAK9 and CAK10) fungal isolates were found to be potent lipase producers out of 14 isolates.

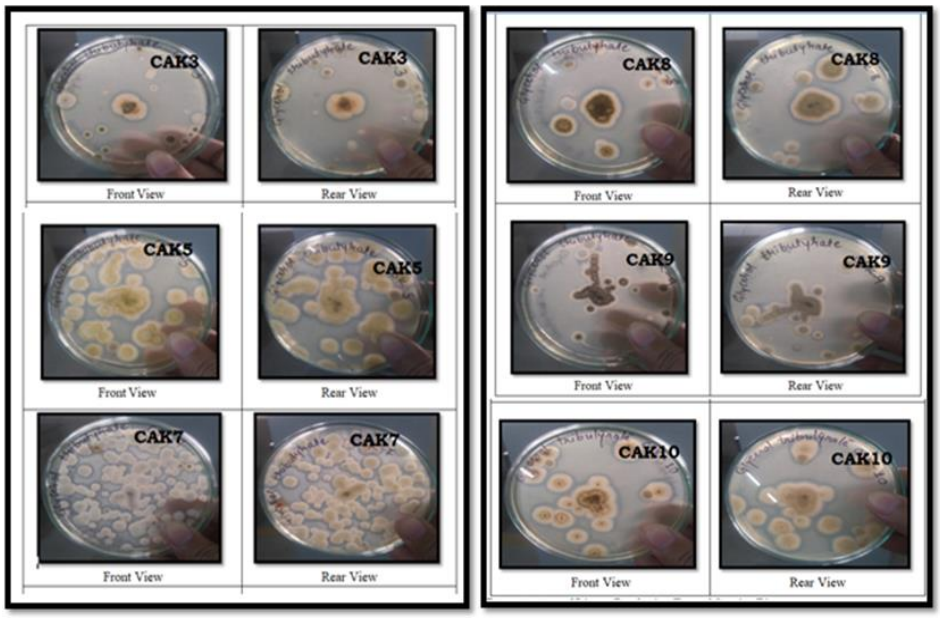


Fig 1: Positive Qualitative Screening by Using Tributyrin agar of Fungal Isolates from Agricultural Soil

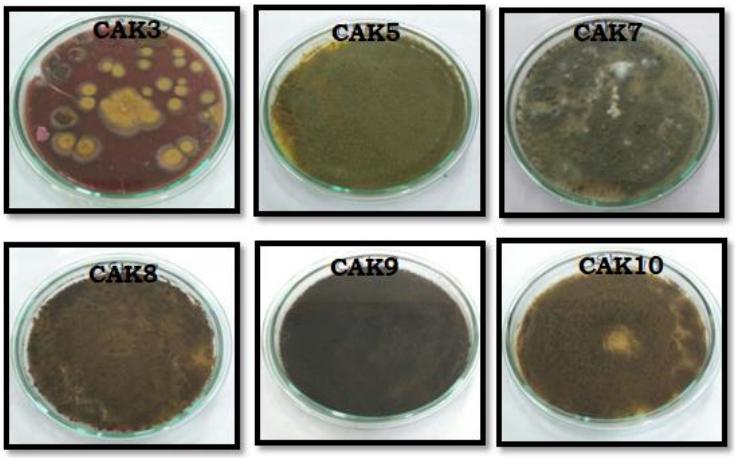


Fig 2: Morphological Characteristics of Fungal Isolates from Agricultural Soil

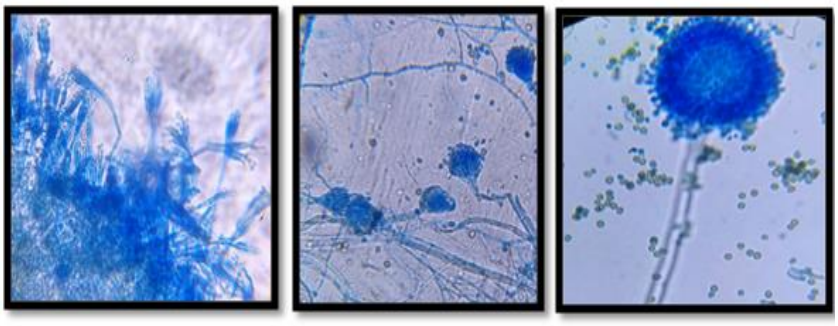


Fig 3: Microscopic Characterization of Fungal Isolates by using Lacto-phenol Cotton Blue Stain

Molecular Identification: After obtaining the potent strains of lipase producers, molecular identification 18S rRNA gene sequencing technology [4] were done by Gujarat State Biotechnology Mission, Gandhinagar, Gujarat. Sequences have been submitted in NCBI for the Accession number. We get the three sequencing and Accession number from NCBI and remaining three are under progressing.

Table 1: Accession numbers of Fungi

Sr.No	Strain_ID	BAB_ID	Accession Number
1	CAK3	7419	MK178552
2	CAK5	7474	MK178553
3	CAK7	7475	MK178554

>SAMPLE_ID_CAK3_BAB_ID_7419_Aspergillus_tamarii

CTCCCACCCGTGTTTACTGTAACCTTAGTTGCTTCGGCG
GGCCCGCCTTTAAGGCCGCCGGGGGGCATCAGCCCCC
GGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCT
GATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGT
TAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGA
TGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATT
GCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACAT
TGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAG
CGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGG
TCGTCGTCCCCTCTTCGGGGGGGACGGGCCCCAAAGG
CAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGG
GCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGC
CGAACGCAAAACAACCATTCTTTCCAGGTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAT

>SAMPLE_ID_CAK5_BAB_ID_7474_Aspergillus_parasiticus

CCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCC
ATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGC
GCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTG
AAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTC
AACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACG
CAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCC
GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCT
GGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCT
GCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCC
CTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGG
CACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCAC
CCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAA
TCAATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGA
TACCCGCTGAACTTAAGCATATCAATAA

>SAMPLE_ID_CAK7_BAB_ID_7475_Aspergillus_tamarii

CCGTGTTTNCTGTAACCTTAGTTGCTTCGGCGGGCCCG
CCTTTAAGGCCGCCGGGGGGCATCAGCCCCCGGGCCC
GCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG
TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACT
TTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGA
ACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAA
TTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCC
CCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATT
GCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGT
CCCCTCTTCGGGGGGGACGGGCCCCAAAGGCAGCGGC
GGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTC
ACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCA
AAACAACCATTCTTTCCAGGTTGACCTCGGATCAGGTA
GGGATACCCGCTGAACTTAAGCATATCAATAA

Effect of pH and temperature on lipase activity:

Lipase activity was maximum by CAK7 (87.2328 ±8.328467 U/ml) i.e. *Aspergillus tamarii* and lowest activity CAK5 (4.185 ± 1.273 U/ml) at the initial pH 8.0, that isolated from agricultural soil sample. Similar study has been done by Supakdamrongkul [9] the lipase activity was maximum shown on pH 8.0, it's firstly reported in biocontrol agent fungus *Nomuraea rileyi.* According to Essamri [10], the highest intracellular lipase activity by *Rhizopus oryzae* at pH 8.5. Nevertheless, generally maximum lipase activity was observed at the initial pH range at 5.0-6.0 this is different from other fungal lipases. Furthermore, lipase activity at the initial pH 8.0 under submerged fermentation was significantly different from lipase activity at other initial pH values according to OFAT.

Ghonemy [23] reported the maximum production of extracellular lipase of *Curvularia* sp. at temperature of 30°C. Mukhtar [20] on his study, he gave the different ranges of incubation temperature from 25°C to 55°C on the productivity of lipase by *A. niger*. Maximum lipase production was achieved at 30°C, followed by 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C. Similar article has been reviewed by Sharma [2], that optimized the production parameters by using different rang minimum 25ºC to maximum 60ºC temperature which had enhanced the production of extracellular lipase from different fungal strains. Production of lipase was observed under submerge fermentation by *Aspergillus niger* in Kilka fish oil as main substrate by Ghasemi [13]. The optimum conditions incubation temperature obtained 3 gr./ml 35°C and apart from 25-45ºC respectively. In recent investigation the Lipase activity is observed at optimum temperature 35°C (291.0590±15.5770 U/ml) irrespective of 20°C to 50°C from *Aspergillus tamarii* under submerged fermentation.

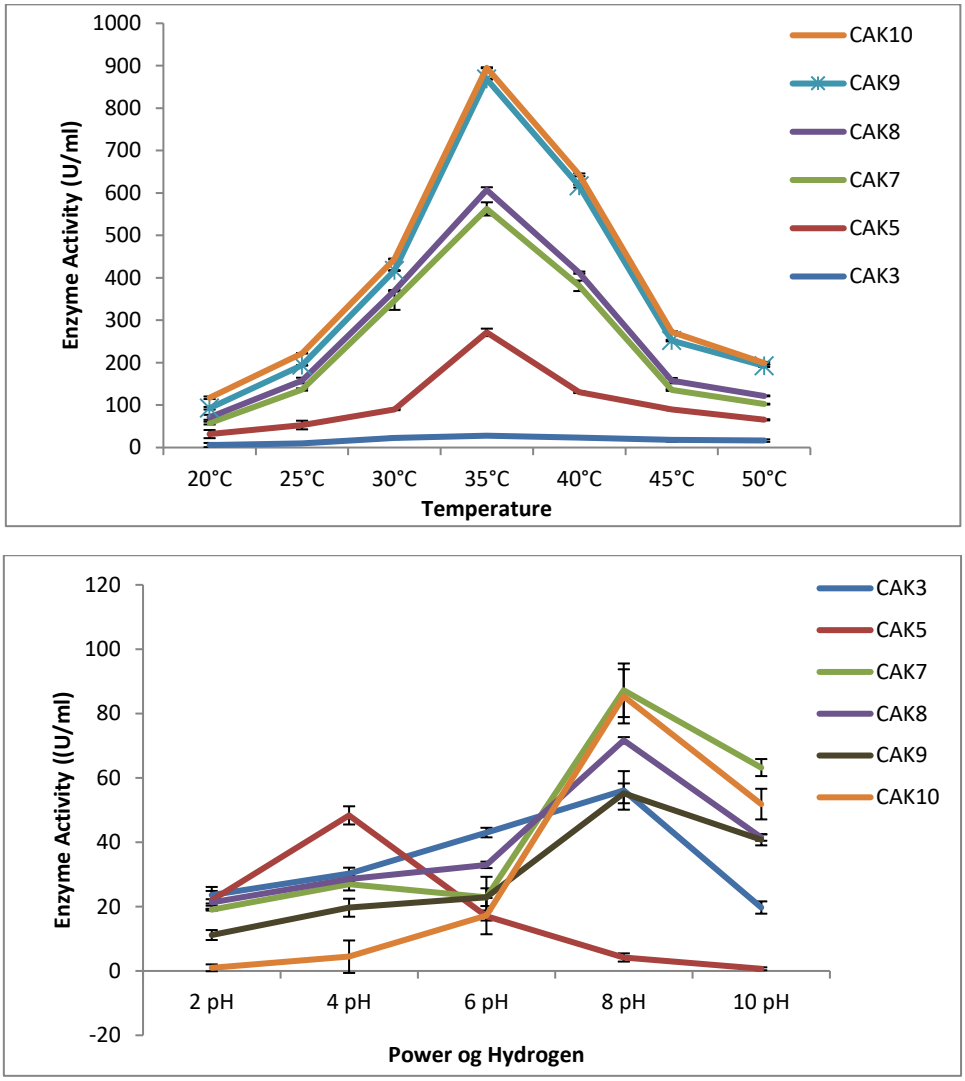


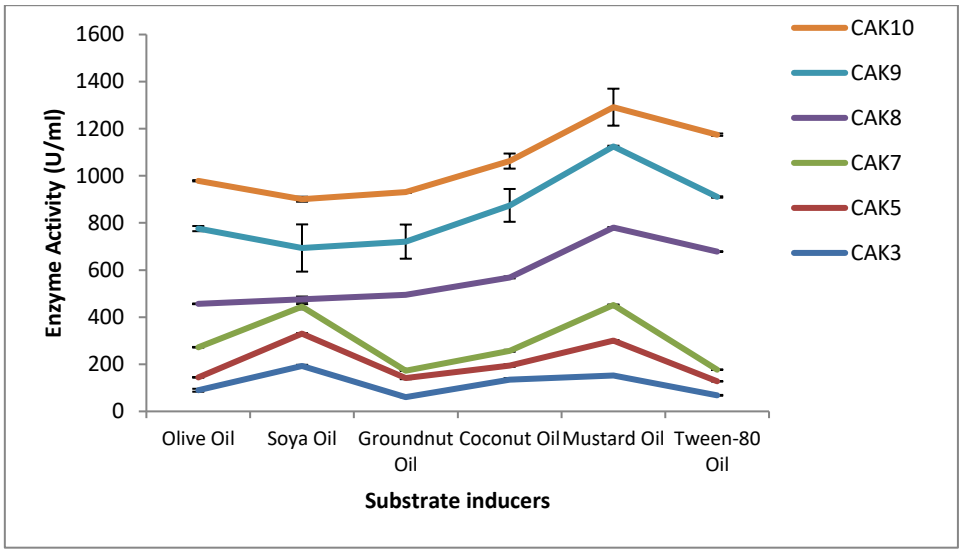
Fig 4: Effect of temperature and pH on lipase activity of various fungal isolates

Effect of incubation time and substrate on lipase activity:

Sumathy [21] in *A. niger* were incubated at 25 °C, 30°C and 37 °C, followed by estimation of activity of lipase after 48 h, 72 h, 96 h and 120 h similar results have been reported by Dheeman [24] several studies involving fungal isolates respectively. Pinheriro [25] in his report maximum lipase production by *Penicillum verracosum* occurred at 96 h. In present study under submerged fermentation optimum lipase activity is shown (109.0713±3.205947 U/ml) at 96 hrs from *Aspergillus tamarii, Aspergillus niger, Aspergillus parasiticus.* The lowest lipase activity is showing CAK3 (84.44 ± 2.27 U/ml) i.e. *Aspergillus tamarii* and highest activity is CAK7 (109.071 ± 3.20 U/ml) i.e. *Aspergillus parasiticus* at the incubation time of 96 hours i.e. 4th day of incubation period.

In the same year of 2011, Nwuche and Ogbonna reported lipase production in twelve fungal isolates belonging to genera *Trichoderma*, *Aspergillus*, *Mucor* and *Penicillium* under Submerged fermentation by using palm oil as carbon supplement. *Tichoderma* sp. (8.24 U ml-1) and *Aspergillus* sp. (7.54 U ml-1) were shown the maximum lipase activity. Sumathy [21] investigated the effect of various concentrations (0.5% and 1.0% v/v) of lipidic inducers (olive oil, gingely oil and mustard oil) on productivity of lipase activity by three different fungi, *A. niger*, *R. oryzae* and *F. oxysporum*. Maximum (6.0 U/ml) enhancement of lipase activity was achieved by *A. niger* with olive oil (1% v/v). Similar report have been shown by Bindiya and Ramana [19], coconut oil, palm oil, cucumber oil, olive oil, mustard oil, sunflower oil and neem oil) were used as a source of substrate inducers on production of lipase by *A. sydowii*. Similar report has been shown our present investigation under submerged fermentation lipase activity has observed into mustard oil (148.44 ± 1.54

to 344 ± 3.22 U/ml) from *Aspergillus tamarii, Aspergillus niger and Aspergillus parasiticus.* The lowest lipase activity is showing CAK5 (148.44 ± 1.54 U/ml) i.e. *Aspergillus parasiticus* and highest activity is CAK9 (344 ± 3.22 U/ml) i.e. *Aspergillus niger* by using mustard oil. Mustard oil is the best carbon sources for the production of lipase enzyme.



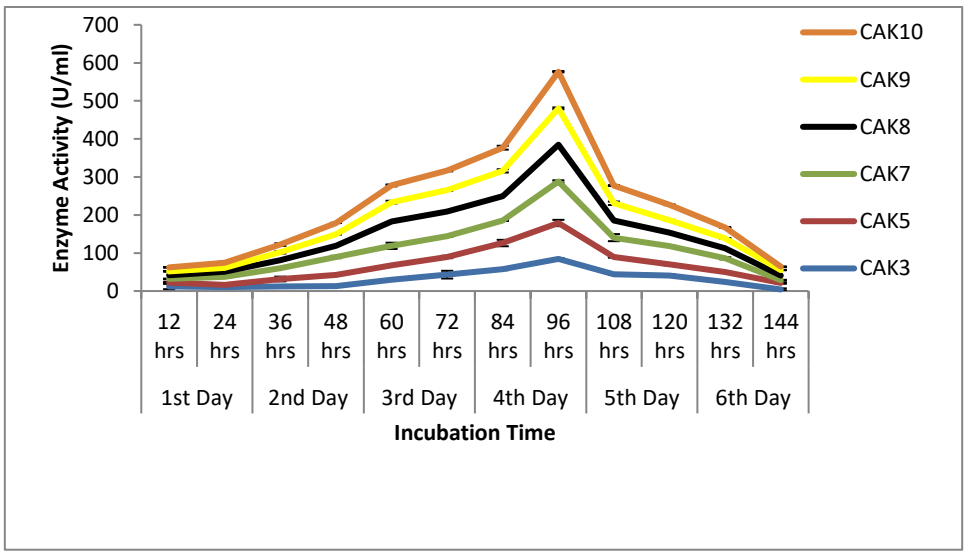


Fig 5: Effect of substrate and incubation time on lipase activity of various fungal isolates

Effect of inoculum volume and agitation on lipase activity:

The role of agitation is also significant in any bioprocess especially in enzyme production. Ire Francis [26] investigated the *Fusarium sp*. Isolated from the soil sucrose, crude oil were employed as nutritional options at pH 2.5 under cultural conditions of 150 rpm agitation rate at 30ºC for 120 hrs (5 days), which yielded enzyme activity, which is 50-60% the total volume of the fermentation broth. Present study has been observed that the optimum lipase activity (688.641±25.679 U/ml) from *Aspergillus tamarii* by using 3.0 ml of inoculum volume in submerged fermentation.

Under the submerged fermentation extracellular lipase was produced by *Aspergillus nidulans* (MBL-S-6). Mubashir [22] studied on different inoculum size, *i.e.,* 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mL were employed to submerged fermentation and to observed effects on lipase production. 2.0 mL inoculum size was optimized for future work. In current study maximum lipase activity was notified at 3.0ml of inoculum volume CAK7 i.e. *Aspergillus tamarii* (191.633 ± 42.86 U/ml).

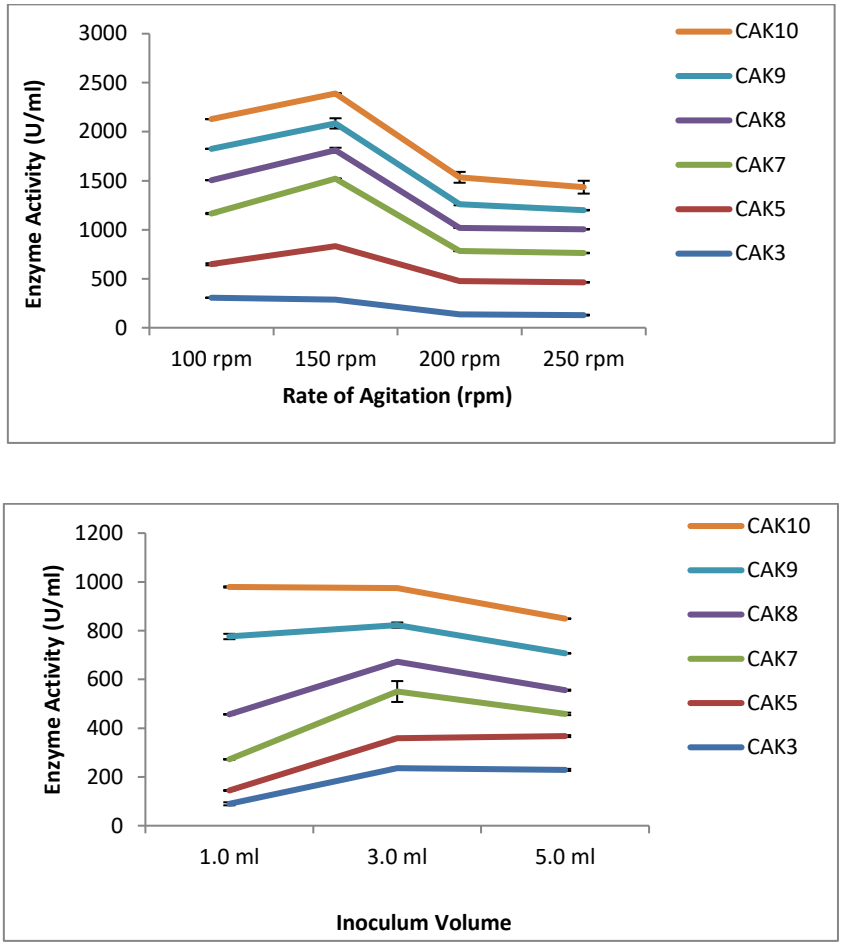


Fig 6: Effect of Agitation and inoculum volume on lipase activity of various fungal isolates

Time Course Study under Optimal Condition:

To check the enhancement of lipase production by using optimized parameters under submerged fermentation the mineral growth medium (MGM) contained (in g/L) NaH_2PO_4: 12.0, KH_2PO_4: 2.0, $MgSO_4.7H_2O$: 0.3 and $CaCl_2$: 0.25. Ammonium Sulphate at 1% and mustard oil - 2% were used as carbon and nitrogen source respectively. The pH 8.0 was adjusted at 35°C incubation temperature up to 96 hours in 3 ml of inoculum volume and 150rpm agitation. Similar media components instead of mustard oil, has been used olive oil by Falony [1].

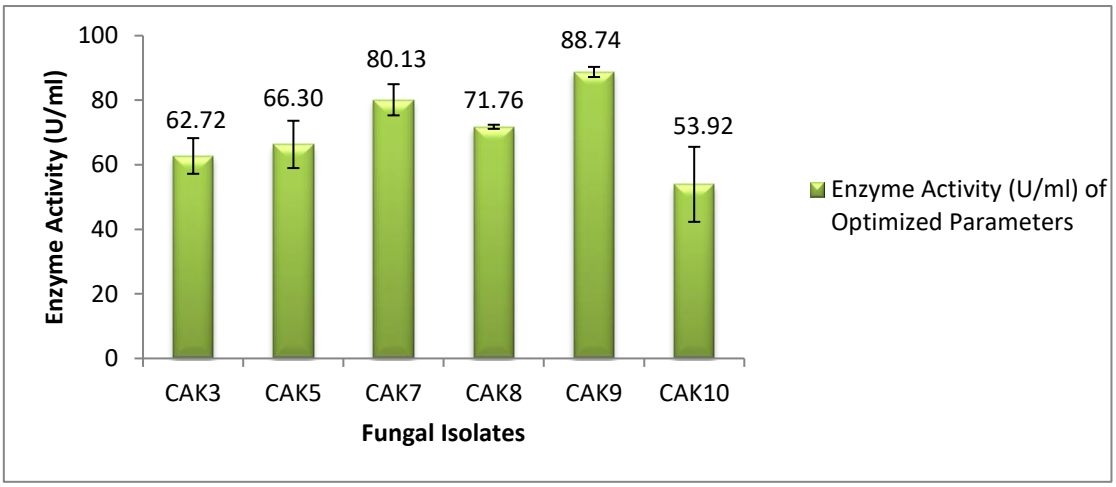


Fig 7: Enzyme Activity of Potent Fungal Isolates from Optimized Parameters under SmF

CONCLUSION

The present investigation revealed that production of extracellular fungal lipases from agricultural (paddy) field of soil sample was found to be enhanced at optimized culture conditions such as medium incubation time, pH, temperature, agitation rate, inoculum volume and various substrate concentrations. From the results, it could be concluded that the medium pH of 8.0 and temperature range of 35°C when incubated up to 96 hours in 3 ml of inoculum volume and 150rpm agitation rate were optimum for maximizing lipase production under submerged fermentation by fungal isolates i.e. mostly of belongs to genera *Aspergillus* sp. The assessment of various substrates for optimizing the production of lipase by potent fungal isolates results revealed that during Submerged fermentation the mineral growth medium (MGM) contained (in g/L) NaH_2PO_4: 12.0, KH_2PO_4: 2.0, $MgSO_4.7H_2O$: 0.3 and $CaCl_2$: 0.25. Ammonium Sulphate at 1% and mustard oil - 2% were used as carbon and nitrogen source respectively. Highest Lipase activity were obtained from CAK7 (*Aspergillus tamarii*) and CAK9 (*Aspergillus niger*). For the production of extracellular lipase under submerged fermentation mustard oil could be used as a substrate inducer would be economically cost effective rather than usage of olive oil that is required for fermentation and to simplify the fermentation media, superior yields and no requirement for complex machineries.

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