STUDIES ON THE OPTIMIZATION OF LIPASE PRODUCTION BY *Aspergillus parasiticus* MK178553 ISOLATED FROM THE PADDY FIELD OF SOIL

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Abstract:
Extracellular lipase producer fungal stain was screened in the laboratory into paddy soil sample collected from Achhoti Village, Kumhari, near Bhilai Region, Chhattisgarh. Identification was done through 18S rRNA as *Aspergillus parasiticus* MK178553. In present investigation the maximum lipase production in shake flask by using submerged fermentation from basal medium. The work has been studied to evaluate the effect of different physical process factors like incubation period, pH, temperature, agitation, and substrate as inducer on lipase production. In mineral Growth Medium by using submerged fermentation showed 4.217 U/ml of lipase activity after 96 h of fermentation at 4 days of incubation period in highly basic pH 8.0 on 150rpm rotation by using shake flask method.

Keywords: Paddy soil, Lipase activity, Submerged Fermentation, *Aspergillus parasiticus*

INTRODUCTION

Lipases are enzymes belonging to the group of serine hydrolases (E.C. 3.1.1.3). Their natural substrates are triglycerides and their mode of action is similar to that of the esterases. However, their activity is considerably increased when they are located at the polar/non polar interface (Maria Gabriela Bello Koblitz *et al.,* 2006). Lipases catalyze the hydrolysis of triglycerides under natural conditions to diglycerides, monoglycerides, fatty acids and glycerol at the oil-water interface, while under certain experimental conditions like in non-aqueous medium catalyze the reverse reaction of esterification (Saxena *et al.,* 2003). Lipases can be found in animal and vegetable cells and they can also be produced by microorganisms. From the industrial point of view these are considered very important, due to their great production potential on a large scale and to the capacity of deterioration of microorganisms (Kirk *et al.,* 2002). Fungal lipases have been studied since 1950s, have presented comprehensive reviews. These lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents.The chief producers of commercial lipases are *Aspergillus niger*, *Aspergillus sps.*, *Candida cylindracea*, *Humicola lanuginosa*, *Mucor miehei*, *Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae*. These reactions are of industrial applications in food processing, organic chemical processing, pharmaceuticals, cosmetics, paper manufacture, and detergent formulations and in environmental management (Sharma A. *et al.,* 2009; Veerapagu M. *et al.,* 2013).

MATERIALS AND METHODS

Collection and processing of soil samples

Soil sample from the regional Paddy field, Achhoti Village, Kumhari, near Bhilai Region, Chhattisgarh was collected for the isolation of fungi. The sample collected was cleaned, dried and subjected for serial dilution technique.

Isolation and basic identification of fungi

Fungi were isolated from the dried soil samples by serial dilution technique on Potato Dextrose Agar Medium – PDA **(**Potato infusion 200 gm, Dextrose 20 gm, Distilled water 1.0 litre and Agar 20-gL^{-1}**)**. Growth of fungi after the incubation of three days at 28±2°C, were identified based on the standard colony characters. The important microscopic features namely Lacto-phenol cotton blue staining, mycelial branching and sporulation pattern of the selected colonies were recorded. The identified colony of fungi was subjected for the molecular characterization and preliminary screening of lipolytic enzyme production. The confirmed isolate of fungi was sub cultured on SDA and preserved at 4°C.

Molecular Identification of Isolated Strain

Sub culturing of isolated fungal strain in SDA medium and incubated 28±2°C for 5 days and to sent the Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, Gujarat for further molecular (18sRNA sequencing) identification.

Qualitative Screening of fungal isolate for Lipase synthesis

The fungal isolate was screened qualitatively by rapid plate assay (Gulati *et al*., 2005) for the synthesis of lipase on Tributyrin Agar Medium- Peptone 0.5% (w/v), Yeast Extract 0.3% (w/v), Tributyrin 0.1% (v/v), Agar 2% (w/v), pH 6.0 for 7 days of incubation at 28±2°C. Selected isolate of fungi was subjected for quantitative screening by broth culture assay (Gwen Falony *et al.,* 2006) using MGM Medium. 10 ml of 0.01% Tween-80 spore suspension of 7 days old test isolate ($13.12x10^{-1}$ spores/ml) was inoculated into 100 ml medium (pH 6.0) and incubated for five to seven days at 28°C.

Production of Lipase by SmF

During the SmF the mineral growth medium (MGM) contained (in gm/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 Olive Oil at 2% were used as nitrogen and carbon sources, respectively. The initial pH was adjusted to 6.0 for fungal culture (Muhannad I. *et al.*, 2011). After incubation the culture was filtrate by using Whatman filter paper no. 1, until the supernatant has been extracted out in the test tube.

Assay of Lipase

Lipase activity was determined by using p-nitrophenyl palmitate (pNPP) (Sigma, USA) as substrate (Ertugrul *et al.,* 2007). The 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 absorbance at λ = 410 nm (UV/VIS spectrophotometer). T

he 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) (ε_{410} = 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 (Muhannad I. *et al.,* 2011). Dissolved protein concentration was determined according to Lowery *et al.,* (1951) method, by using bovine serum albumin as a standard.

Effect of Physical Process Optimization Parameters of Lipase Activity

Effect of incubation time

Optimal incubation time for maximal enzyme production was determined by incubating the inoculated media for a total period of 144 hours and analysing the samples at regular interval of 12 hours for enzyme

activity. Medium preparation, inoculation and incubated at 28°C, lipase activity was measured spectrophotometrically against control.

Effect of incubation temperature

To ascertain the optimum temperature for the enzyme activity, the assay mixture was incubated in the temperature range of 20°C to 50°C (20°C, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C) for 4 days and assayed for the lipase activity by spectrophotometric method.

Effect of pH on lipase activity

Initial pH of the medium that could support maximal enzyme production was determined by adjusting the pH of the medium to various levels i.e., 2, 4, 6, 8 and 10 with either 1 N HCl or 1 N NaOH and determining the enzyme activity after 4 days of incubation by spectrophotometric method.

Effect of agitation on lipase activity

Lipase production was studied by incubating the inoculated media taken in the conical flasks in shake flask conditions at different rpm (100, 150, 200 and 250) and enzyme activity was assayed after 4 days of incubation by spectrophotometric method.

Effect of substrates on lipase activity

Ideal substrate that induces maximal lipase production was studied using different oil substrates (2% conc.). Six different refined oils, viz, olive oil, soya oil, groundnut oil, coconut oil, mustard oil and tween-80 were used for the study. After 4 days of incubation enzyme activity was estimated by spectrophotometric method.

RESULTS & DISCUSSIONS

Table 1: Qualitative screening of lipase producing fungal strain

Substrate	Organism CAK5 - Zone of Hydrolysis (cm)		
	3rd Day	5th Day	7th Day
Glycerol Tributyrate	3.5	3.6	3.7

Fig 1. ***Aspergillus parasiticus*** **(Front)**

Fig 2. ***Aspergillus parasiticus*** **(Back)**

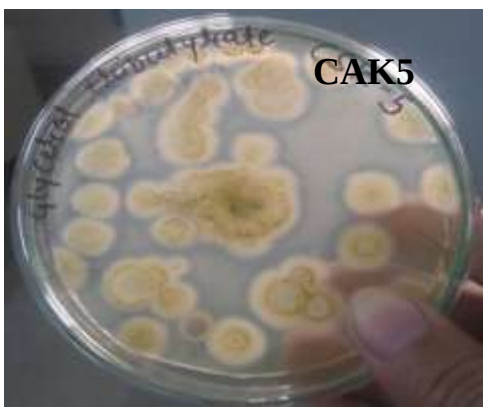


Fig 3. *Aspergillus parasiticus* (Front) **Fig 4. *Aspergillus parasiticus* (Back)**

Isolation of Selected Fungal Isolate

Generally, fungi used to grow in mesophilic environment within a temperature rang 5-35°C. In the recent study, the 8 isolates of fungi being isolated from agricultural field of Paddy field, Achhoti Village Kumhari, near Bhilai Region, Chhattisgarh are also representing the same mesophilic nature of the isolate fungi. Morphological identification by visualization of colony characteristics and lacto phenol cotton blue staining generally provides a better preliminary level of understanding to tentatively identify any fungal genera based on fair comparison with standard taxonomic keys with respect to fungal taxonomy classification (Costa *et al.,* 1999). In the present study, the diversity in pigmentation of colonies and accumulation of mycelia in agar plates in myriad form, besides lacto phenol cotton blue staining has helped to identify preliminarily out as 8 fungal isolates few fungal isolates are CAK5 *Aspergillus parasiticus.* Similar kind of data had also been reported by Hoog *et al.,* 2000. The report also emphasized on shape, size, arrangement and phalides, conidiophore, Conidiospore and phialospore as important characteristic taxonomic identification key to tentatively identify any fungal genera. The similar report on species of *Aspergillus; Penicillum;* also reported by Ibrik *et al.,*1995; Toida *et al.,* 1998; Costa *et al.,* 1999.

Identification of Strain CAK5

After sequence similarity analysis of obtained sequence (Figure 5) with NCBI sequence data, the strain had a homology of more than 100% with *Aspergillus parasiticus* (MK138351.1) with comparison of the 18s rRNA sequencing. Therefore, the strain belonged to *parasiticus* species of *Aspergillus* genus and named *Aspergillus parasiticus (BAB_ID* 7474 *Accession Number* MK178553).

>SAMPLE_ID_CAK5_BAB_ID_7474_Aspergillus_parasiticus

CCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAG
CCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGA
TTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACG
CAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCA
CATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCAC
GGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCA
CCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGC
CGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATCAATAA

Figure 5: 18s rRNA sequence

Qualitative Screening of Lipase Producing Strain

Cleared zone (3.7cm) was shown by *Aspergillus parasiticus* at 7th day of incubation, the clearing zone of hydrolysis is seen as a result of lipase action on the tributyrin agar medium. The screening is dependent on media composition especially on inducer which is mostly lipid or allied substrate like oil, environmental condition; physiological parameters such as pH, NaCl concentration, temperature (Treichel *et al.,* 2010). In the present study, tributyrin agar media has greatly influenced on the selected potent fungal isolate for synthesis of lipase. Niches out of 8 total isolates activity is recorded A*spergillus flavus* followed by

identified fungal strain *Penicillum verrucosum* and the learnt activity is recorded by *Aspergillus niger*. Similar kind of report is also there by Aravindan *et al.,* 2007. There are various reports citing occurrence of lipase by *Aspergillus* more than any other filamentous fungi.

Effect of Physical Process Parameters on Lipase Activity

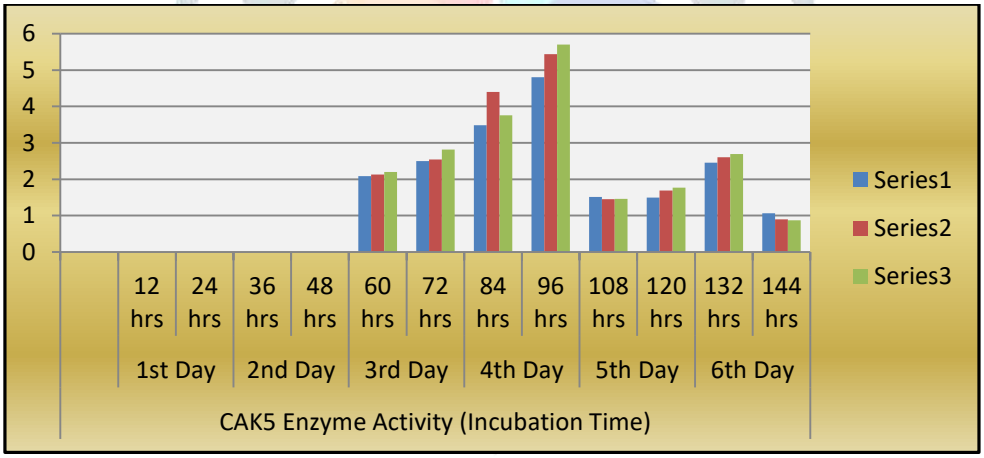
Optimization of culture parameters is one of the best strategies for enhancing microbial enzyme production and is often achieved by studying the production medium composition. The physical process parameters always a great impact on fluctuation of enzymes. The all total five physical process parameters namely; Incubation time (Days), Incubation temperature, pH, Agitation and Substrate inducers. In the present investigation has been evaluated to check the effect on enzyme activity. In lipase biosynthesis, an incubation period of 4 days under submerged fermentation was found to be optimum for enhanced lipase production by *Aspergillus parasiticus* MK178553. Similar observation has seen on *Rhizopus oryzae* ZAC3 (Zainab A. *et al.,* 2017). Most of lipase producing organisms grows in moderate temperature between 25 and 40, making them mesophilic in nature. Optimum temperature for lipase production by *Aspergillus parasiticus* MK178553 was observed at 35°C when incubated at different ranges of temperatures 20 to 50°C. Lipase activity was enhanced on optimum pH 8.0 by using substrate as inducer source mustard oil at 150 rpm in submerged fermentation.

Quantitative Screening of Lipase Producing Strain

Report of Falony G. *et al.,* (2006) has reported during the SmF by using mineral growth medium (MGM) lipase activity of *Aspergillus niger was* 1.46 IU/ml. In the present study, in a range of *Aspergillus parasiticus* MK178553 4.217 U/ml enzyme activity of lipase has been depicted which is correlating with the available data.

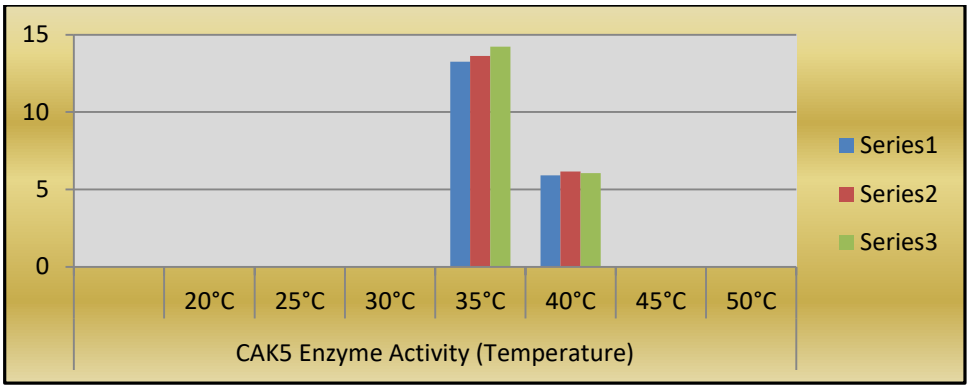
Effect of Physical Process Parameters of Lipase Activity

Effect of Incubation Time (Days)



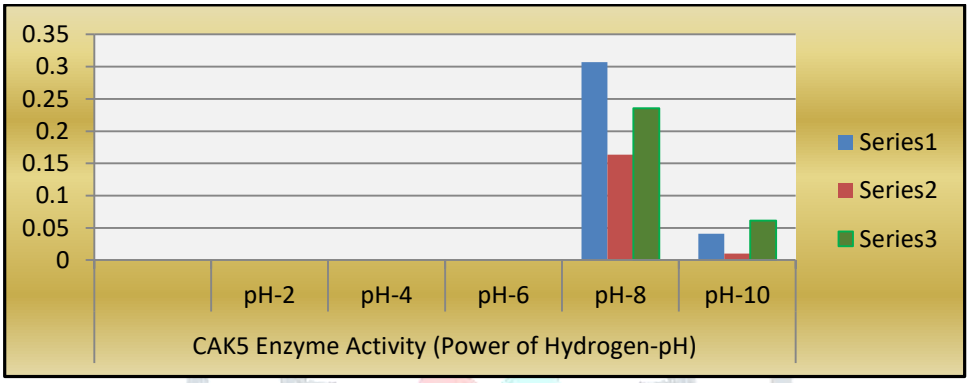
Graph 1: Effect of incubation time on lipase activity

Effect of Incubation Temperature



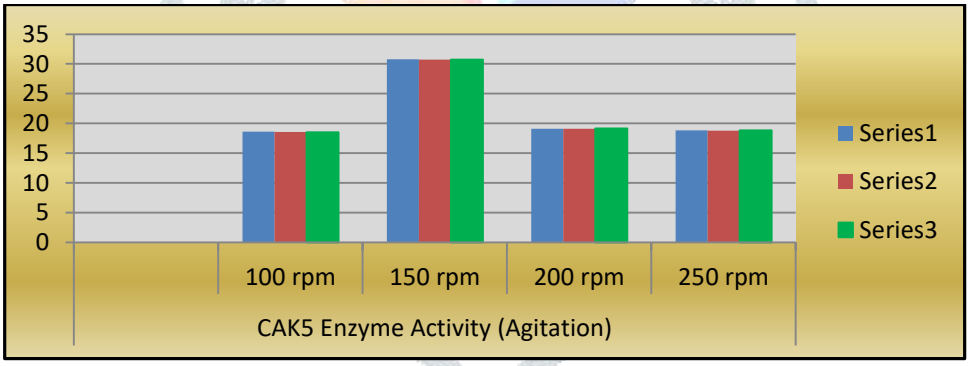
Graph 2: Effect of incubation temperature on lipase activity (triplicate)

Effect of different pH



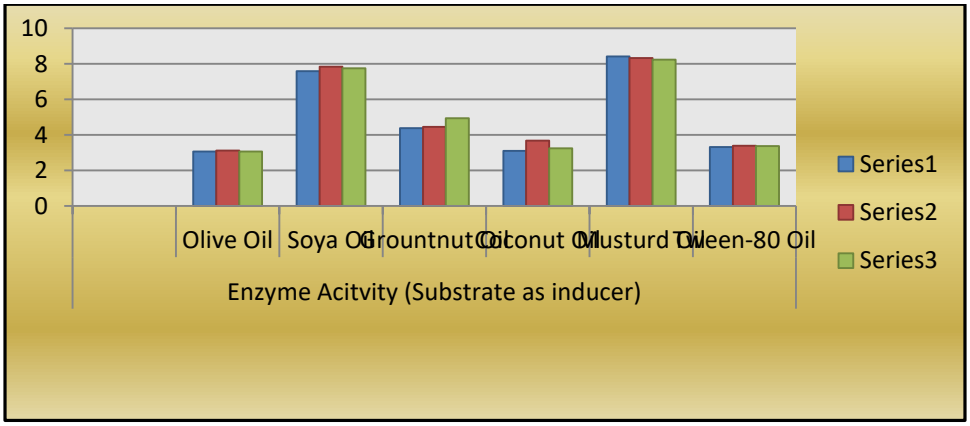
Graph 3: Effect of pH on lipase activity (triplicate)

Effect of Agitation



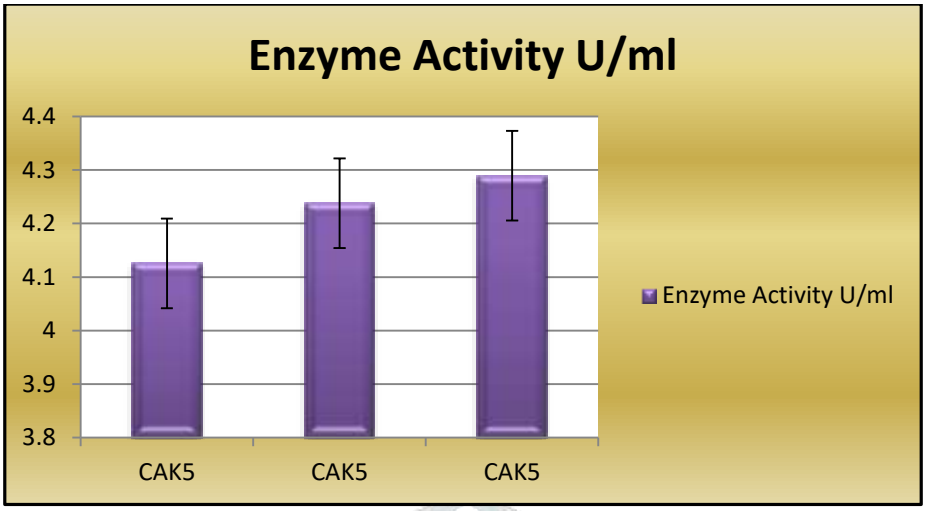
Graph 4: Effect of agitation on lipase activity

Effect of different Substrates Inducers



Graph 5: Effect of different substrates inducers on lipase activity

Lipase Activity with Optimized Parameters by Using SmF



Graph 6: Lipase activity of *Aspergillus parasiticus* in basal (MGM) medium by SmF

CONCLUSION

There for maximum production of enzyme with high activity is still an important filed of investigation. Maximum production of industrially important enzymes like lipase through media engineering is still holding its place. Lipase is stable at basic conditions and mordent temperature was isolated was observed in culture conditions greatly influenced lipase production and optimization of these culture parameters improved lipase production. The organism (*Aspergillus parasiticus* MK178553) can be biotechnologically exploited for commercialization due to its unique features. Similarly best environment factors like time, temperature, agitation, pH and substrate also showed positive effect on lipase production. Results observed that mustard oil is a good source of substrate inducers it enhance the lipase activity.

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