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# Production of lipase from *Azadirachta indica* oil seed cake using solid state fermentation

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**Abstract**
*Azadirachta indica* (Neem) seed cake is obtained as a by-product from Neem oil industry. The current study was focused on the solid-state fermentation of *Azadirachta indica* oil seed cake to produce commercially important extracellular lipase enzyme using isolated fungi species. The *Penicillium species* was isolated and further screened for extracellular lipase production. The lipase activity in the crude was found to be 2.74 U/ml/min. The pH-6.5 of the culture medium exhibited maximum lipase production. Further, the enzyme activity at different pH and temperature conditions revealed that the optimum pH is 7.5 & optimum temperature is 30 ˚C. The enzyme was further concentrated and fractionated by ammonium sulphate precipitation. Among different saturations the maximum enzyme was recovered at 80% saturation. The dialysed enzyme sample was immobilized using sodium alginate gel and the entrapped enzyme exhibited the lipase activity of 0.533U/ml/min. The Gas Chromatography profiles of transesterified samples indicated the effectiveness of enzyme catalysed transesterified samples (4h & 8h) over the base catalysed sample, with the drastic reduction in free fatty acids content indicating high conversion of fatty acids into their respective methyl esters.

**Keywords:** Lipase, Neem oil seed cake, *Penicillium,* Transesterification, *Pongamia*, Biofuel

## 1. Introduction
*Azadirachta indica* seed cake is a low-value by-product resulting from bio-diesel production. The seed cake is highly toxic, but it has great potential for biotechnological applications as it comprises of bioactive molecules that could be having beneficial application in agriculture, medicine, animal feed and other industries. Lipases (triacylglycerol ester hydrolases, E.C. 3.1.1.3) are the enzymes that catalyse the hydrolysis of triglycerides to fatty acids and glycerol. The economical method resulting in lipase production is termed as solid state fermentation (SSF). The SSF technique involves the use of a solid culture medium as a nutrient source and support for microorganism growth. Several studies have been carried out to obtain lipases by solid state fermentation SSF using different agro-industrial residues, such as wheat bran, gingelly oil cake, rice husks, castor bean waste (Toscano *et al*., 2011) [11]. Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomyce (Toscano *et al*., 2011) [11]. Fungal lipases are of great concern for large scale production. These lipases are being used extensively due to their low cost of extraction, thermal and pH stability, group substrate specificity, and action in organic solvents. Oil cakes provide ideal nutrient support in SSF representing both carbon and nitrogen sources. Oil cakes /oil meals are the byproducts obtained after oil extraction from the seeds. Using fungal species, oil cakes have been reported to be good substrate for economical enzyme production (Ramachandran *et al*., 2007) [8]. In view of the above facts, there is a great urge to explore novel lipases of industrial uses. The present study reports isolation and production of a lipase producing fungal strain from Neem seed cake enriched soil and to evaluate its enzymatic potential in transesterification for use in biodiesel production.

## 2. Materials and methods
### 2.1 Soil sample collection & Enrichment
The Soil samples were collected from oil contaminated area in a depth of 2 – 5 ft, in sterile plastics bags and was enriched for the lipase production by mixing the soil with Neem oil seed cake followed by daily sprinkling of the mixture of tween 80 and olive oil over a period of 10-15 days.

### 2.2 Isolation of Lipase Producing Microorganisms

The enriched soil sample (one gram) was suspended in 250ml Erlenmeyer flask containing 100ml of sterile saline further subjected for sequential serial dilution. Approximately, 100µl of each dilution was inoculated on Rose Bengal Agar (MRBA) plates by spread plate technique and plates were incubated at 28 ˚C for 3-4 days and further the isolated fungal colonies was sub-cultured and subjected to qualitative screening for identification of extracellular lipase producing micro-organisms using rhodamine olive oil (ROA) agar plates (Savitha *et al*., 2007) [9].

### 2.3 Lipase medium assay

Lipase producing fungal species produce a zone of clearance (hydrolysis) when the isolated fungal strains was spread on the lipase inducing medium containing peptone, 5g; yeast extract, 3g; sodium chloride, 5g and agar-agar, 20g; with the 50 ml Egg Yolk Emulsion as lipase inducer in one litre. The zone of hydrolysis was measured after 24 - 72 h of incubation at 28 °C.

### 2.4 Rhodamine-olive oil-agar plate assay (ROA)

A sensitive and specific plate assay for detection of lipase producing microorganisms makes use of rhodamine-olive oil. The assay was carried out according to method described by Savitha *et al*., (2007) [9].

### 2.5 Morphological characterization of isolated fungal species

The identification of fungal isolate was carried out by microscopic analysis using lactophenol cotton blue stain. The fungus was identified on the basis on the morphology of colony, mycelia and spore characteristics.

### 2.6 Culture conditions

The identified fungal species were cultured in 250 ml flasks containing 20g *Azadirachta indica* seed cake as solid support impregnated with a concentrated broth, in a ratio of 1g /1.5 ml, of nutrient source modified culture medium (SSF Medium) consisting of $KH_2PO_4$ ( 1.0g/l), $MgSO_4$ (0.5g/l), KCl (0.5g/l), $FeSO_4$ (0.01g/l), Dextrose (12.5g/l), Peptone (20g/l), Tween-80(15 ml/l), Triton x 100(4 ml/l) and Olive oil(25 ml/l). The initial pH of the medium was adjusted to pH 6.5 and then sterilized in autoclave at 121 ˚C for 1h, allowed to cool to room temperature.

### 2.7 Solid State Fermentation (ssf)

SSF was carried out using Azadirachta indica seed cake. Then, suitable volume of spore suspension was inoculated into the SSF media containing flasks and incubated at 28- 30 ˚C for a period of 10 days (Balaji *et al*., 2008) [2].

### 2.8 Enzyme extraction

After 7 -10 days of incubation the extracellular lipase enzyme was extracted by adding Sodium phosphate buffer 20 mM (pH-8.0) containing 1% (W/V) Triton X100. The flasks were kept in a rotary shaker at 60 rpm for 1h at 25 ˚C. The ingredients of the flask were subsequently filtered and the culture filtrate was used as the crude enzyme source.

### 2.9 Enzyme Assay /lipase assay (Titrimetric method)

The lipase activity was measured using modified titrimetric method as described by Borkar *et al*. [2]. The reaction mixture contains 10 ml of olive oil containing 1% (v/v) tween-80, 4ml of 0.1M sodium phosphate buffer (pH 7.0), 500µL of 2% calcium chloride and 1ml of enzyme. The total contents were incubated at 37 ˚C in water bath for 20 mins with frequent shaking for every 5 min intervals. The Reaction was terminated by the addition of 20ml of acetone:ethanol mixture (1:1 v/v). The reaction mixture was titrated against 0.1N NaOH. The lipase activity was calculated using the following formula:

Lipase activity = (Test-Control) × Normality of NaoH×100 / Incubation Time (min).

=..........U\ml\min

**Unit Activity**: One unit of lipase activity was defined as the amount of enzyme liberating one Micro mole of fatty acid per minute under standard assay conditions.

### 2.10 Optimization of fermentation condition

#### 2.10.1 Effect of pH on lipase production:

The effect of pH on lipase production was performed by varying pH of the SSF medium from 5 to 8 whereas the other parameters were unaltered. At the end of fermentation time period of 10 days, crude enzyme was extracted by mixing the fermented substrate with 100 mL of phosphate buffer (20mM; pH-8.0) and the mixture was kept on orbital shaker at 60rpm for 1h at 25 ˚C. The obtained extract was filtered and the supernatant was used as crude enzyme source for lipase assay.

#### 2.10.2 Determination of effect of Temperature and pH on enzyme activity

The temperature and pH optimum of extra cellular lipase assay was carried at different temperatures ranging from (20,28,30,37,40,45,50,60,70,80 & 90 ˚C). The optimal pH was determined by incubating the enzyme- substrate at various pH from 5 to 8 using different buffers, citrate buffer (pH 5.0 & 5.5), sodium phosphate buffer (pH 6.0,6.5 & 7.0) and Tris-HCl buffer (pH 7.5 and 8.0).

### 2.11 Partial purification

The crude lipase obtained was subjected to protein fractionation by 80% ammonium sulphate saturation. The pellet was collected by centrifuging at 10,000 rpm for 20 min at 4 °C, dissolved in 50 mM phosphate buffer (pH -8.0) & dialysed against 10 mM Tris-HCl buffer (pH 8) for 24 hours, with three changes of buffer. The dialyzed enzymatic fraction was used for protein estimation and enzyme immobilization studies (Shafei *et al*., 2010) [10].

### 2.12 Protein estimation

The protein content of the enzyme was determined according to the Lowry ‘s method (Lowry *et al*., 1951) [7].

### 2.12 Immobilization of partially purified lipase

**Calcium alginate beads**

One ml of enzyme was mixed with 10 mL of sodium alginate solution and then the mixture was stirred thoroughly to ensure complete mixing. Further the solution was dropped into chilled 0.1M $CaCl_2$ solution with help of a dropper. Ca-alginate beads were formed. After 20 min of hardening the beads were collected by filtration, then washed with sodium phosphate buffer (0.1 M, pH-8.0) several times to remove the unbound enzyme.

### 2.13 Effect of incubation time on enzyme activity
The immobilized enzyme beads obtained are subjected to lipase assay to determine the enzyme activity at different incubation time periods. The optimal time period for effective enzyme activity was evaluated by incubating the immobilized enzyme beads along with substrate at various time intervals for 1h, 2h and 4h at 37 ˚C.

### 2.14 Transesterification process
**Base catalysed Transesterification process**
The oil was heated to around 120̊C to remove moisture content. Then the solution was cooled down to 60 ˚C and 20% methanolic KOH in 6:1(methanol:oil) ratio was added slowly for 2h with constant stirring and allowed to cool. Then the solution was transferred to separating funnel and allowed to stand. The two top layer obtained was crude diesel and lower layer as glycerol. The top layer was evaluated for presence free fatty acids contents.

### 2.15 Enzyme catalyzed transesterification process
The reaction process involved pretreatment of crude pongamia oil and Production of Biodiesel by Batch Process at 4h & 8h reaction time, with conditions for the The enzymatic transesterification reaction to obtain biodiesel was carried out in two batches containing 5% enzyme concentration, methanol: pongamia oil ratio (3:1), one batch was incubated for 4 hours and other for 8 hours at 37ºC temperature and 100rpm agitation speed. The Crude biodiesel product obtained was purified with n-hexane and was subjected to TLC and GC analysis

### 2.15 Qualitative and quantitative analysis
#### 2.15.1 TLC analysis
The biodiesel produced by two batches with (4h & 8h reaction time) was analyzed by Thin Layer Chromatography (TLC) using TLC Silica gel 60 F254, 25 Aluminum sheets 20×20 cm (Merck), the developing solvent used was a mixture of toluene-chloroform-acetone (7:2:1, v/v/v) and resolved components on TLC plates were visual by iodine vapours.

#### 2.15.2 Preparation of sample for GC analysis
0.5g of sample was taken, to this 4pellets of KOH, 5ml of methanol was added heated until KOH dissolved completely. To the above mixture add 10ml of water and sonicated followed by boiling for few minutes along with 0.5ml of Conce $H_2SO_4$. The solution was allowed to cool was extracted with 10ml of n-hexane. The extracted n-hexane layer was filtered through sodium sulphate and finally sample was injected into the GC system.

#### 2.15.3 GC conditions
The identification of Fatty acids in the biodiesel produced was done using GC analysis. The column of GC was Zebron wax plus (gradient mode) and film thickness was 0.25 micrometer. Nitrogen gas was used as a carrier which had a flow rate of 0.25ml/min. The injector and detector temperature was set as 250 °C for 10 mins respectively. The run time was set as 50 mins. The sample used for the detection was diluted with n-hexane and out of which 0.1µl sample was injected into the system with a split ratio of 5:1.

## 3. Results and discussion
### 3.1 Screening & isolation of fungal strains
The four different fungal strains were isolated and screened for the production of lipase from enriched soil sample. The isolated fungal strains were identified as *Peniciilium, Trichoderma, Aspergillus* and *Cladosporium* species (fig-1). These strains further screened for the production lipase by growing them on rhodamine olive oil agar media. Among these strains *penicillium* species exhibited extracellular lipase activity with the formation of orange fluorescent halos around the fungal colonies when it was observed under the UV light (fig-2). Similar fluorescent zones were also seen in the earlier report (Jette and Ziomek., 1994) [6] in which Rhodamine fluorescence-based assay was used to screen 32 fungal species from diverse sources, resulted in the effective screening of fungi species (Savitha *et al*.,2007) [9].

### 3.2 Morphological characterization & Identification of Isolated species
The microscopic examination carried out by lactophenol cotton blue staining procedure revealed the morphological feature of mycelia and spore characteristic. Green coloured fungal colony possessing a white mycelia border around the colony. The green coloured spores were observed on the surface of the colony. On the basics of micro and macro morphological nature of the fungal isolate, comparing it with the Manual of Soil Fungi by Gilman. It was tentatively identified as *Penicillium* species (figure-3).

### 3.3 Lipase activity assay
The crude extracellular lipase activity was determined as 2.74U/ml/min by titrimetric method. Similar results were reported in case of lipase production by *Aspergillus niger* using SSF media where in different substrates such as wheat bran and soyabean meal was used and the 2.9 U/ml lipolytic activity was observed in this case (Aunstrup *et al*., 1979) [1].

### 3.4 Effect of pH on enzyme production
To optimize the pH condition for the maximum lipase production using SSF was carried out under varied pH conditions between 5.0 to 8.0. Maximum production of lipase was fund to be at pH 6.5 (0.4U/ml/min). So the present study revealed that the lipase production by *penicillium* sp. was induced more under mild acidic growth environment (figure-4). Similar result was documented in case of *P. chrysogenum* with the maximal lipase production between pH 6.5–7.0 (Shafei *et al*., 2010) [10].

### 3.5 Determination of effect of pH and temperature on enzyme activity
Extracellular lipase assay was carried out under different pH conditions. According to the study the maximum enzyme activity was observed at pH 6.5 and (figure-5). The lipase activity was found to stable up to pH-7.5 and enzyme activity was drastically reduced at pH-8.0.This result is in accordance with the earlier data of lipase from *F. solani* FS1which maintained around 80% of its initial enzyme activity when incubated for 1h at alkaline pH (7.2 - 8.6), with a decrease in lipase activity at pH beyond this range. The major cause for the drastic drop in the enzyme activity in alkaline pH condition mainly due to the alkali mediated denaturation of enzyme protein.
The lipase assay was carried out at different temperatures (20-90 $^0$C). The maximum lipase activity was observed at temperature 30ºC (figure-6). Further increase in the temperature has drastically declined enzyme activity due to thermal denaturation of enzyme. The results obtained was

similar to the other fungal lipase enzymes including *Fusarium solani* (Optimum temperature 30 °C) (Bhatti *et al*., 2007) [4].

### 3.6 Partial purification of extracellular lipase by ammonium sulphate precipitation

The crude lipase was subjected to different ammonium sulphate fractionations (40%, 50%, 60%, 70%, 80% and 90%). The maximum lipase was precipitated at 80% saturation and the enzyme activity was found to be 9.9U/ml/min (table-1). The protein content was measured in crude enzyme preparation and partially purified enzyme sample found to be 6mg/ml and 3.26mg/ml respectively.

### 3.7 Effect of immobilization on the enzyme activity

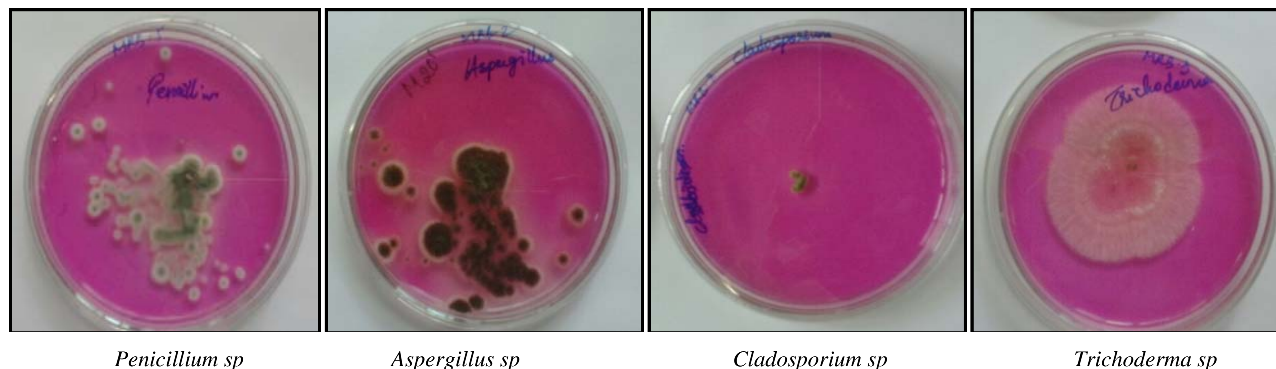
Lipase assay when carried out with immobilized enzyme beads with different incubation periods and using two different substrates such as olive oil & pongamia oil. The immobilized enzyme exhibited maximum activity with pongamia oil upon 1 hour incubation when compared to that of 2h and 4h incubation period. Enzyme activity declined as the incubation time was increased (Table-2). This is because of inactivation of enzyme. Similar results were reported when Plant Lipase from *Pachira aquatic* was immobilized in Alginate Beads. The enzyme activity had gradually declined after 1h of incubation (Barbara *et al*., 2014) [3]. The result also emphasise that the pongamia oil could be the suitable substrate over olive oil. The activity of immobilized enzyme after 1h of incubation time period for pongamia oil was found to be 0.533U/ml/min and for olive oil found to be 0.49 U/ml/min.

### 3.8 TLC analysis

TLC analysis of crude oil, base catalysed and enzyme catalysed samples revealed the conversion of free fatty acid (figure-7)

### 3.9 GC Analysis

GC was performed to identify and quantify the fatty acids content present in the crude oil, chemical and enzyme catalysed transesterified oil samples. The four major fatty acids component found in the crude pongamia oil were palmitic acid, oleic acid, linoleic and linolenic acid during the GC analysis. The further the quantification of these fatty acids in all the three samples was summarized below in Table-3. The fatty acids content was found to be high in crude pongamia oil gradually decreased after chemical mediated transesterification process (figure-8and9). Whereas alignate immobilized enzyme mediated tranesterified samples (differenent time incubation 4h and 8h) showed a drastic reduction of free fatty acids content indicating that most of their free fatty acids were effectively converted into their respective methyl esters (figure-10 and 11).



*Penicillium sp* *Aspergillus sp* *Cladosporium sp* *Trichoderma sp*

**Fig 1:** Fungal strains isolated from enriched soil and grown on MRBA media

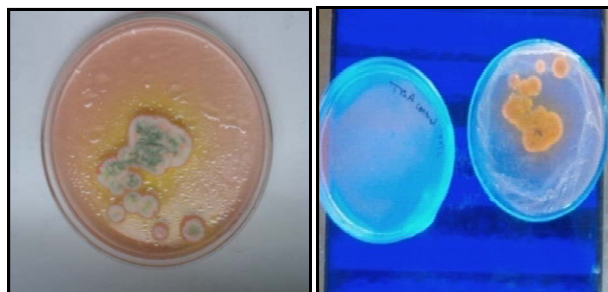


**Fig 2:** Pure culture of *Penicillium sps* grown on rhodamine assay plate

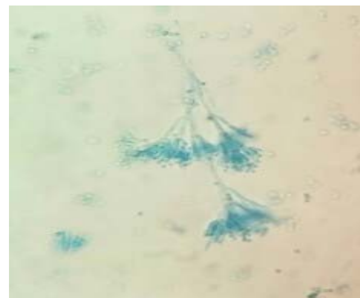


**Fig 3:** Microscopic image of *penicillium* sps under 40x magnification

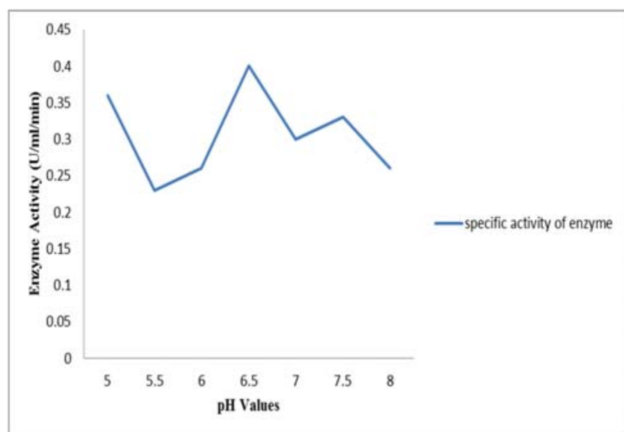



**Fig 4:** Optimisation of pH culture conditions for the production of lipase

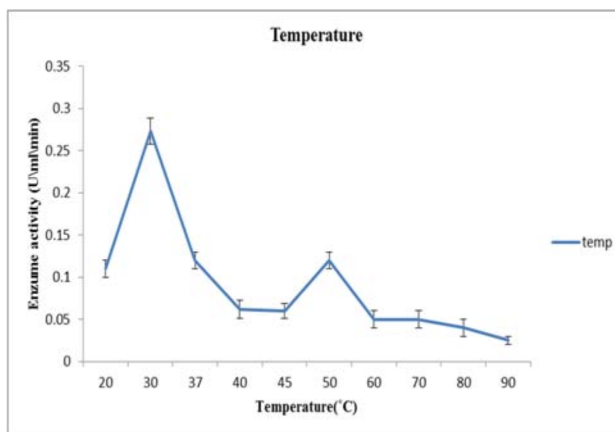



**Fig 6:** Effect of Temperature on enzyme activity

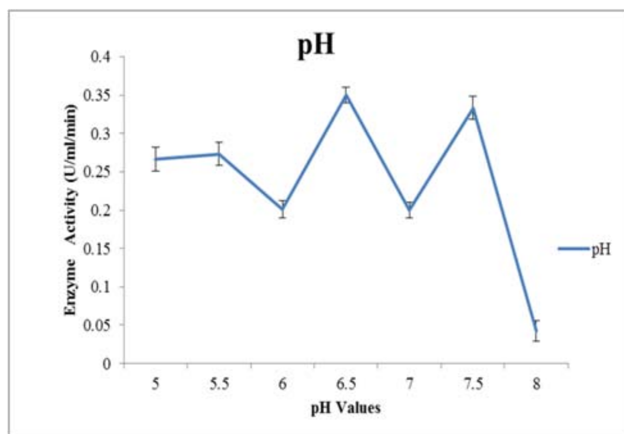



**Fig 5:** Effect of pH on enzyme activity

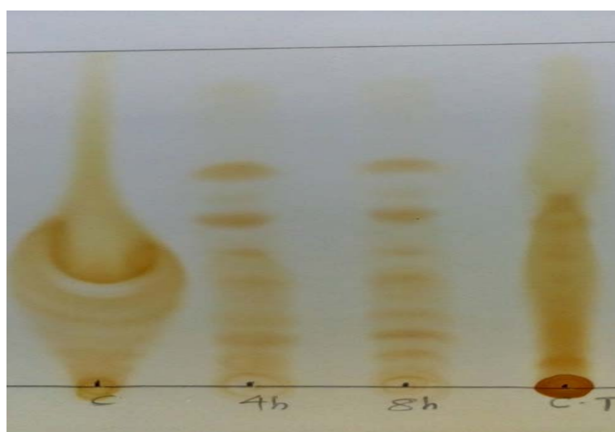
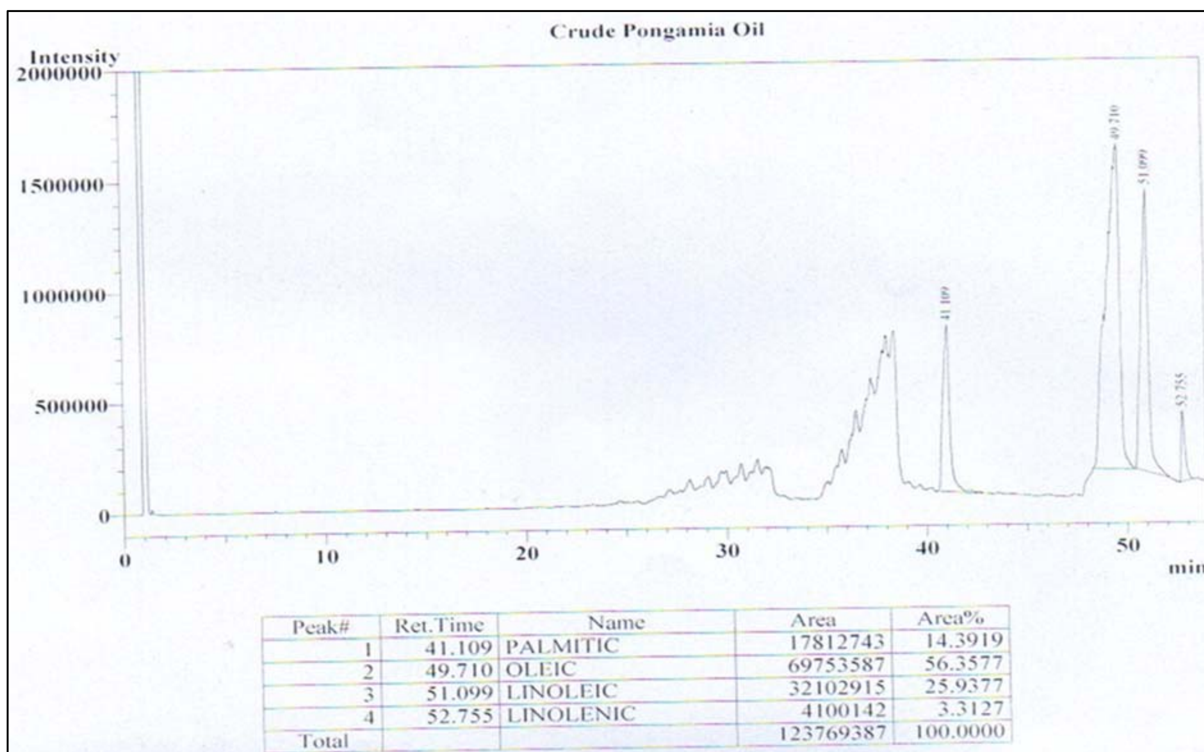
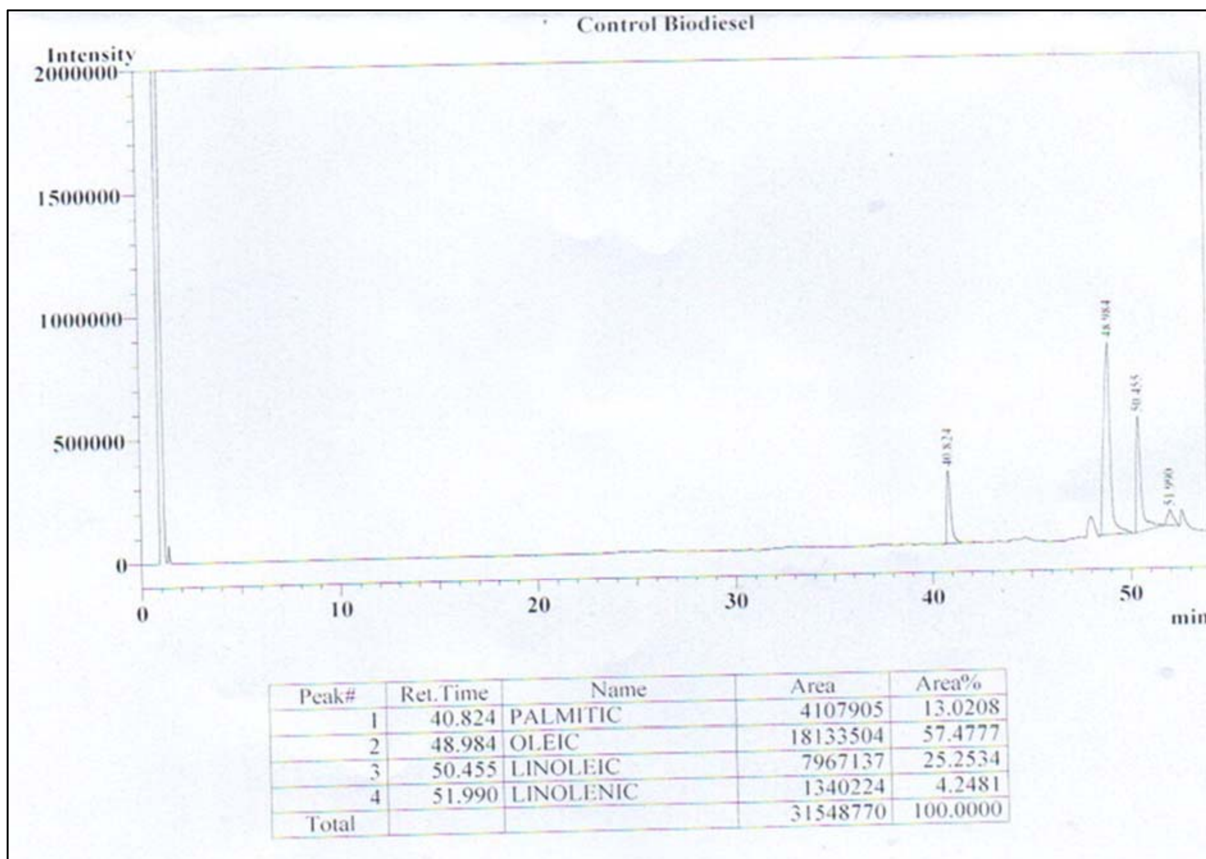



**Fig 7:** TLC analysis of transesterified samples.C-crude pongamia oil diluted with chloroform(1:1),4h & 8h-Enzyme catalysed transesterified samples,C.T-Base catalysed transesterified control sample



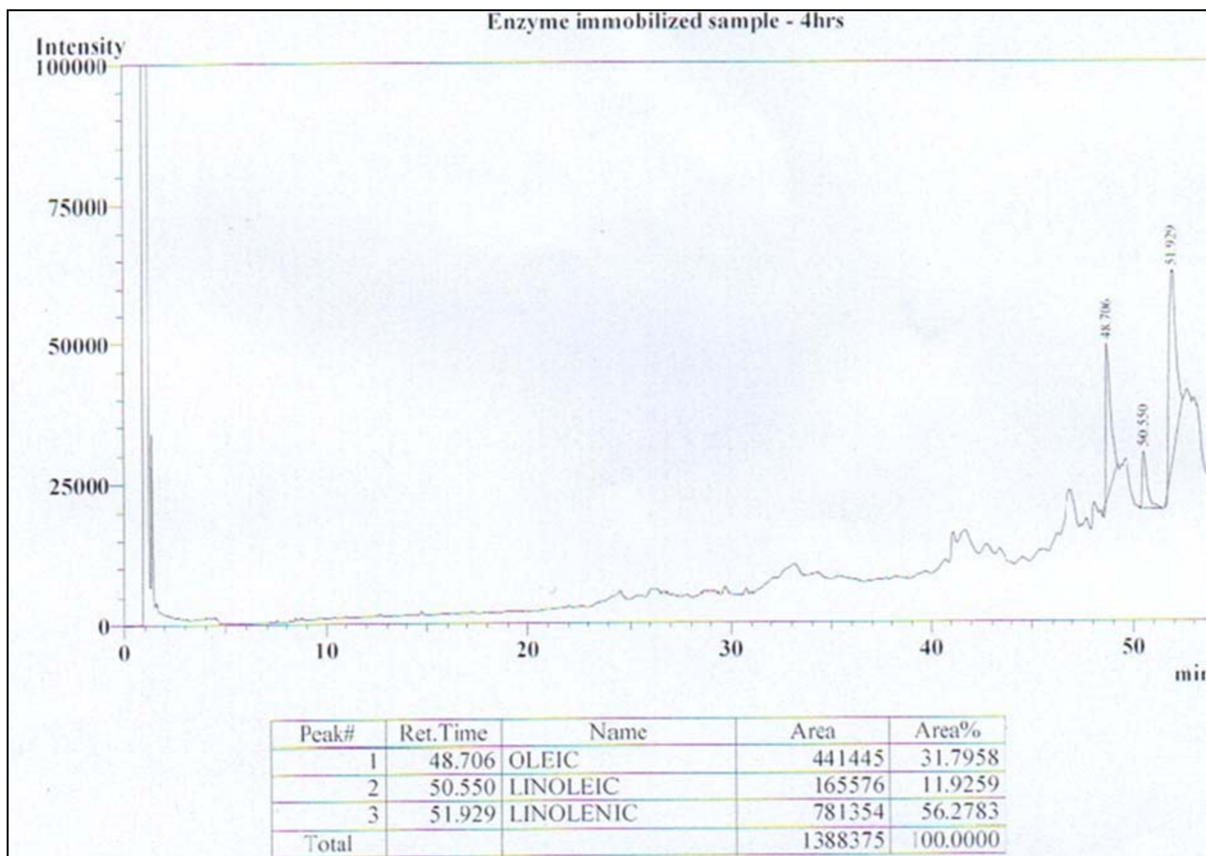

| Peak# | Ret.Time | Name | Area | Area% |
|---|---|---|---|---|
| 1 | 41.109 | PALMITIC | 17812743 | 14.3919 |
| 2 | 49.710 | OLEIC | 69753587 | 56.3577 |
| 3 | 51.099 | LINOLEIC | 32102915 | 25.9377 |
| 4 | 52.755 | LINOLENIC | 4100142 | 3.3127 |
| Total | | | 123769387 | 100.0000 |

**Fig 8:** GC chromatogram for fatty acid composition in crude pongamia oil



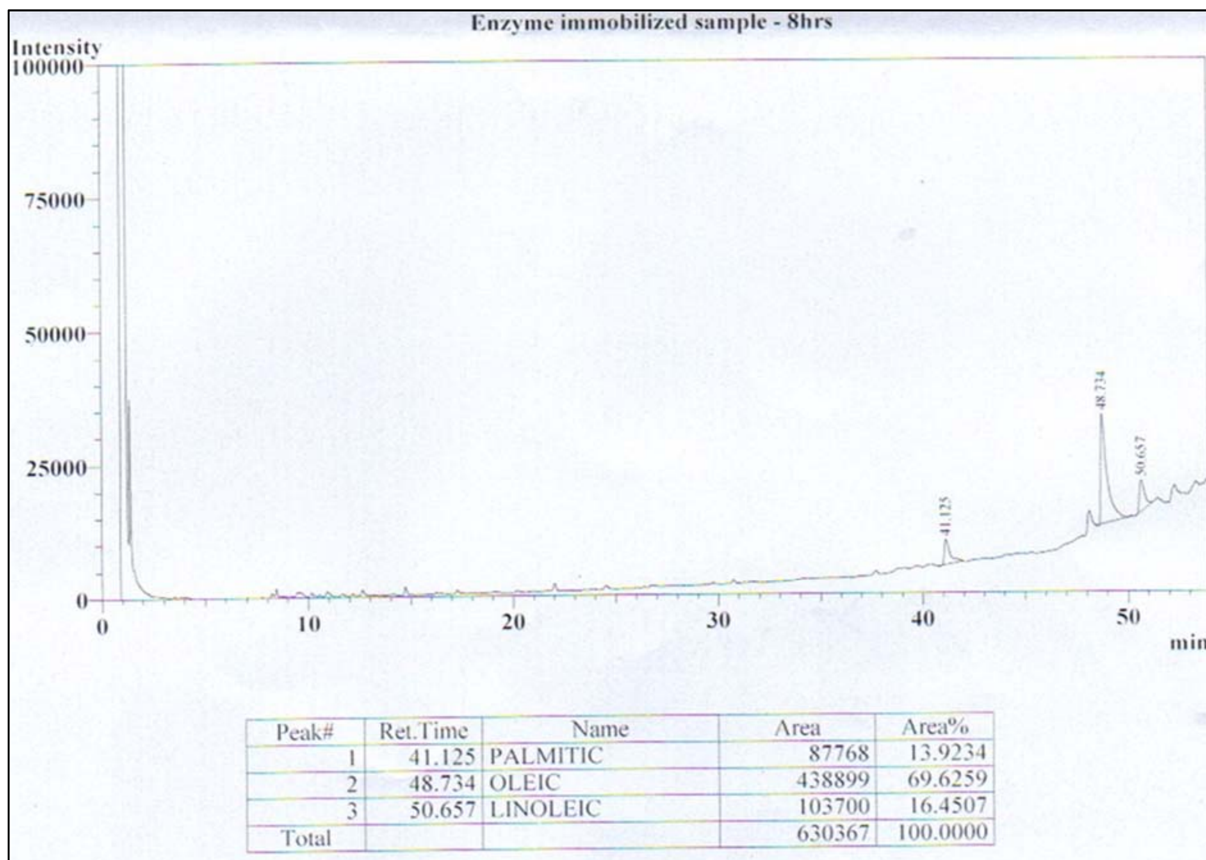

| Peak# | Ret.Time | Name | Area | Area% |
|---|---|---|---|---|
| 1 | 40.824 | PALMITIC | 4107905 | 13.0208 |
| 2 | 48.984 | OLEIC | 18133504 | 57.4777 |
| 3 | 50.455 | LINOLEIC | 7967137 | 25.2534 |
| 4 | 51.990 | LINOLENIC | 1340224 | 4.2481 |
| Total | | | 31548770 | 100.0000 |

**Fig 9**: GC chromatogram for fatty acid composition in KOH catalysed transesterified sample




| Peak# | Ret.Time | Name | Area | Area% |
|---|---|---|---|---|
| 1 | 48.706 | OLEIC | 441445 | 31.7958 |
| 2 | 50.550 | LINOLEIC | 165576 | 11.9259 |
| 3 | 51.929 | LINOLENIC | 781354 | 56.2783 |
| Total | | | 1388375 | 100.0000 |

**Fig 10:** GC chromatogram for fatty acid composition in enzyme catalysed transesterified sample-4h




| Peak# | Ret.Time | Name | Area | Area% |
|---|---|---|---|---|
| 1 | 41.125 | PALMITIC | 87768 | 13.9234 |
| 2 | 48.734 | OLEIC | 438899 | 69.6259 |
| 3 | 50.657 | LINOLEIC | 103700 | 16.4507 |
| Total | | | 630367 | 100.0000 |

**Fig 11:** GC chromatogram for fatty acid composition in enzyme catalysed transesterified sample-8h

**Table 1:** Partial purification of extracellular lipase by ammonium sulphate precipitation

| Saturation level (%) | Enzyme activity(U/ml/min) |
|---|---|
| 40 | 1.86 |
| 50 | 5.33 |
| 60 | 6.43 |
| 70 | 6.8 |
| 80 | 9.9 |
| 90 | 7.93 |

**Table 2:** Effect of incubation time on immobilized enzymes

| Samples | Enzyme Activity (U/ml/min) | | |
|---|---|---|---|
| | Incubation Time Period (Hours) | | |
| | 1 h | 2 h | 4 h |
| Pongamia Oil+Phosphate Buffer | 0.533 | 0.2 | 0.1 |
| Olive Oil+Phosphate Buffer | 0.49 | 0.3 | 0.08 |
| Olive Oil | 0.4 | 0.22 | 0.1 |

**Table 3:** GC chromatogram for fatty acid composition of crude oil, chemically transesterified desile and Biodesile

| Fatty acids (%) | Crude oil | Control | 4h | 8h |
|---|---|---|---|---|
| Palmitic | 14.4 | 3.3 | ----- | 0.07 |
| Oleic | 56.4 | 14.8 | 0.1 | 0.4 |
| Linoleic | 25.9 | 6.5 | 0.4 | 0.08 |
| Linolenic | 3.3 | 1.1 | 0.6 | ------ |

## 4. Conclusion

The present study explores an agro- industrial residue (Neem oil seed cake) which has the potential to produce lipase in an economically feasible manner by a new promising fungal strain of *Penicillium* species. Fairly high level of lipase activity 2.74U/ ml/min was achieved in 10 days time period of solid state fermentation at 30 °C when supplemented the basal medium with 1% olive oil. Olive oil supported good growth and increased lipase activity significantly. So, olive oil was found to act as an inducer of lipase production for *penicillium* strain. Lipase activity with optimized growth condition led to enhancement in lipase production with optimum pH as 6.5 at 30 ˚C. Further the stability studies revealed that the enzyme was highly stable at pH 6.5, was fairly stable upto pH 7.5 and temperature stability was upto 30˚C.This indicated the alkaline nature of lipase enzyme Also alkaline lipases acts as an ideal choice for application in detergent. Later on immobilization studies employing lipase enzymes entrapped in calcium alginate beads with use of pongamia oil as substrate resulted in high enzyme activity of 0.533U\ml\min, during the 1h incubation time period. The Gas Chromatography profiles of transesterified samples indicated the effectiveness of enzyme catalysed transesterified samples (4h & 8h) over the base catalysed sample, with the drastic reduction in free fatty acids content indicating high conversion of fatty acids into their respective methyl esters.

## 5. Acknowledgement

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