Lipase-producing *Aspergillus niger* LP4 Isolated from Banana Plantations: Lipase Production Optimization *via* Central Composite Design and Environmental Applications

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GRAPHICAL ABSTRACT

Lipase-producing *Aspergillus niger* LP4 Isolated from Banana Plantations: Lipase Production Optimization *via* Central Composite Design and Environmental Applications

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Lipases degrade triglycerides and are used in detergent, biodiesel production, and chemical industries. In this work, lipase-producing fungal strains were enriched. A total of 10 morphologically different fungi were isolated and screened for lipase production. The isolated indigenous *Aspergillus niger* LP4 utilized a mixture of *Calophyllum inophyllum* oilcake and coconut oil cake (1:1), showing greater lipase production (127.5 ± 5.5 U/g substrate) than *Calophyllum inophyllum* oil cake (120.2 ± 3.4 U/g substrate) and coconut oil cake (103 ± 1.8 U/g substrate). A one-variable-at-a-time approach revealed optimum pH at 6.5 (139.2 ± 4.5 U/g substrate), 30 °C (152.4 ± 7.3 U/g substrate), 6% (v/w) inoculums (174.1 ± 5.4 U/g substrate), and 60% moisture content (180.5 ± 3.3 U/g substrate). After screening bioprocess variables by the traditional method, the selected three variables (pH, inoculum concentration, and moisture level) were optimized by the central composite design experiment. The central composite design gave 2.1-fold more lipase production compared to an unoptimized medium. The F-value of the designed model was 12.98, and the p-value was 0.0002. In this model, the terms A, B, C, A2, and C2 were significant model terms. The crude lipase showed exceptional compatibility with detergents, improved wash performance, and released free fatty acids from the wastewater.

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Keywords: Calophyllum inophyllum oil cake; Coconut oil cake; Solid-state fermentation; Fungi; Lipase

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INTRODUCTION

Bioproducts prepared from renewable sources are a desirable alternative to fossil fuels and help achieve a sustainable future for nature. Several varieties of biomass are utilized as feedstocks for product formulation, including aquatic plants, agricultural wastes, marine food waste, and crops (Sathya *et al.* 2023). The production of products from biomass, especially industrial residues or agricultural wastes, helps to produce sustainable and economical products (Kumar and Kim 2018). There are several agriculture residues used as feedstocks for enzyme production; however, there has not been much consideration of oil cakes as substrates for lipase production. *Calophyllum inophyllum* oilcake is a non-edible, inexpensive, and renewable carbon source used as the substrate. This plant is

cultivated in sub-tropical and tropical regions. *C. inophyllum* seeds have been used as the feedstock to produce biodiesel. The oil cake contains a rich source of carbon and energy. *C. inophyllum* oilcake was previously used to produce various products. *C. inophyllum* oilcake was used as the substrate to produce PHA and H_2 using bacterial species such as *Rhodobacter sphaeroides* and *Enterobacter aerogenes* (Arumugam *et al.* 2014). Coconut oil is extracted from dried copra, and coconut oil cake (COC) is a major oily byproduct. Coconut oil cake is used as a feed ingredient for ruminants; otherwise, it is considered waste. It is rich in soluble sugars, starch, lipids, proteins, and nitrogen. Coconut oil cake was used as the substrate to produce various bioproducts, including α-amylase (Ramachandran *et al.* 2004), lipase (Gowthami *et al.* 2015; Venkatesagowda *et al.* 2015; Prabaningtyas *et al.* 2018), and L-asparaginase (Priya *et al.* 2012; Ghosh *et al.* 2013) by bacteria, fungi, and actinomycetes (Al Farraj *et al.* 2020; Arokiyaraj *et al.* 2024).

Agro-industrial wastes are used as substrates for the biosynthesis of enzymes or products, and solid-state fermentation is widely used for this purpose. The filamentous fungi have the potential to penetrate the substrate and utilize solid media for energy and product formation(Vijayaraghavan and Vincent 2014; Colla *et al.* 2015; Chen *et al*. 2024). The utilization of agricultural residues reduces environmental pollution; otherwise, these residues cause environmental pollution and greenhouse gas emissions. Thus, utilizing these agricultural residues will reduce environmental pollution and the production cost of enzymes. The amount of enzyme production varies based on the substrate type and available nutrients, including carbon, nitrogen, and energy. The selection of suitable substrate material directly influences enzyme yield (Pallín *et al.* 2024). Agro-wastes, such as soybean cake, wheat bran, gingelly oil cake, rice bran, sugar cane bagasse, and oil cake, have been used (Fleuri *et al.* 2014). On an industrial scale, lipases can be produced by submerged fermentation. This method requires sophisticated equipment and synthetic culture media and increases the possibility of microbial contamination. Solid-state fermentation requires less energy, increased product recovery, high yield, and the solid substrate supports anchorage to the microbial cells (Ashok *et al.* 2017; Al-Ansari *et al.* 2020; Oiza *et al*. 2022; Boondaeng *et al.* 2024). In this study, the authors used agricultural residues for the biosynthesis of lipase using *C. inophyllum* oil cake, coconut oil cake, and neem seed oil cake in SSF. In the next step, various combinations of agricultural residues were used, and the suitable combination (*C. inophyllum* oil cake and coconut oil cake) was selected. Then, the physical factors, such as moisture, pH, temperature, and inoculum concentration, were optimized by the traditional method, followed by the response surface methodology. Then, the crude lipase was applied for industrial and environmental applications.

EXPERIMENTAL

Isolation of Lipase-Secreting Fungi

A soil sample (1 kg) was collected from the agricultural field. The soil sample was enriched with potato dextrose broth containing 1% (v/v) olive oil. The mixture was stirred at 110 rpm at 28± 2 °C for 4 days. After 4 days of enrichment, the viable fungi were transferred to freshly prepared potato dextrose agar medium and incubated for 7 days, and morphologically different colonies were isolated.

Screening of Lipase-Producing Fungi

The potato dextrose agar (PDA) medium (Himedia, Mumbai, India) containing $CaCl_2 \cdot 2H_2O$ (0.1) was sterilized at 15 lbs for 15 min. After sterilization, the medium was cooled and supplemented with polysorbate 80 (Tween-80) (1%, v/v). The isolated fungal strains were streaked on PDA plates and incubated for five days at 28± 2 °C. Lipase-producing strains were identified based on the precipitation reaction of calcium in the medium by the activity of fatty acids. The selected fungi were cultured in potato dextrose broth medium and incubated at 28 °C for five days. After five days of culture, the mixture was centrifuged at 5000×g for 10 min. Enzyme assay was performed and the result was expressed as U/mg.

Characterization of Fungus

The isolated fungus, which showed maximum lipase production, was selected for characterization studies. Briefly, the fungal strain LP4 was cultured in potato dextrose broth medium for 3 days at 28± 1°C. Then, the genomic DNA was extracted with the cetyltrimethylammonium bromide (CTAB) extraction method (Stewart 1997). The internal transcribed spacer (ITS) region of the fungus was amplified using standard ITS1 and ITS4 primers as described previously using DreamTaq PCR Master Mix (Thermo Scientific, Waltham, MA, USA) (White *et al.* 1990) based on the manufacturer's instructions. The ITS regions were amplified and sequenced, and the sequences were classified using the BLAST tool using the NCBI fungal ITS sequence databases. The characterized fungus showed 99.14% similarity with *Aspergillus niger* and was named *Aspergillus niger* LP4. DNA sequences (684 bp) were obtained and submitted to GenBank databases, and accession numbers were assigned.

Substrates

C. inophyllum oil cake, coconut oil cake, and neem seed oil cake were collected from the local market. These oil cakes were used as the substrate to produce lipase in SSF. These cakes were dried and powdered mechanically, and the particle size ranged between 1 and 1.5 mm.

Inoculum

The screened fungal strains were inoculated individually in a conical flask containing 100 mL of potato dextrose broth (Himedia, Mumbai, India). The culture was incubated for five days at 28± 1 °C and was used as the inoculum.

Solid State Fermentation

Ten grams of oil cakes were transferred into an Erlenmeyer flask, and the initial pH of the medium was maintained using 0.1 M phosphate buffer (pH 6.5). The moisture content (60%) was adjusted using phosphate buffer, which ensured there was no free water in the Erlenmeyer flask. The Erlenmeyer flasks were sterilized for 30 min at 15 psi. It was cooled and inoculated with 5% inoculum on the solid substrate. Then the mixture was incubated at 30 °C for five days, and lipase production was observed.

Enzyme Extraction

The fermented substrate with the selected fungal biomass was withdrawn after five days. The fermented substrate was mixed with 100 mL of double-distilled water containing 1% Triton X-100. The mixture was shaken on a rotary shaker at 200 rpm/min, and the

solution was filtered using Whatman No. 1 filter paper. The supernatant was centrifuged at 5000×g for 10 min. The crude enzyme sample was used for the enzyme assay.

Lipase Assay

The lipase activity of the sample was assayed. Briefly, 1.0 mL of culture filtrate was mixed with an olive oil emulsion (5 mL) and incubated for 30 min at 30 ± 1 °C. This sample and substrate mixture were quenched (20 mL) using an acetone-ethanol (1:1) mixture. Then, the mixture was titrated against 0.05 N NaOH until it became a pink color, and the amount of fatty acid was determined. To the blank, inactive enzyme was added. The enzyme activity was expressed as U/g dry substrate.

Effect of the Combinations of Oil Cakes for Lipase Production

C. inophyllum oil cake (CiOC), coconut oil cake (COC), and neem seed oil cake (NSOC) were prepared in different combinations. The selected combinations were 1:1 (CiOC, COC), 1:1 (CiOC, NSOC), 1:1 (COC, NSOC) and 1:1:1 (CiOC, NSOC, COC). These substrate combinations were selected to screen for the optimum concentration to improve lipase yield. This experiment was performed in triplicate, and the result was expressed as the mean± SD.

Optimization of Physical Factors on Lipase Production

C. inophyllum oil cake (CiOC) and coconut oil cake (COC) mixtures were used as substrates for lipase production unless otherwise stated. To optimize the effect of pH on lipase production, the pH of the medium was adjusted using buffer at various pH values (5.5 to 7.5); the other factor was the optimum level (5 days of incubation). To screen the effect of temperature on lipase production, the culture was inoculated into the medium and incubated for 25 to 45 °C. The other factors were the optimum level (5 days of incubation) and pH 6.0. To screen the initial moisture content of the medium, the flasks containing medium were maintained at different moisture levels (40% to 80%), and the other factors were the optimum level (5 days of incubation, pH 6.0, and 30 °C incubation temperature). To screen the optimum inoculums level, the flasks containing medium were sterilized and inoculated with various concentrations of inoculums (2 to 10%, v/w). The result was expressed as mean ±SD.

Statistical Optimization of Lipase Production

The variables such as pH (X1), inoculum level (X2), and moisture content (X3) were selected based on a one-variable-at-a-time approach (Table 1). *C. inophyllum* oil cake and coconut oil cake mixture were used as the substrate for SSF until otherwise stated. The central composite design model comprised 20 experimental runs for the three selected variables. The experiment was designed using Design Expert version 7.0 software (StatEase, Minneapolis, MN, USA). The experiment was performed in duplicate, and the response (lipase activity) was fitted into a second-order polynomial equation by a multiple regression method. The designed response surface model was explained by the following Eq. 1,

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ii} x_i x_j \quad (1)$$

where Y is the predicted lipase activity; $\beta$is the interactive term; β_i is the linear coefficients; β_{ii} is the quadratic coefficients; β_{ij} is the interactive coefficients; and x_i and x_j are the independent variables.

The three-dimensional response graphs were plotted using Design Expert software. The lack of fit of the model was analyzed, and an insignificant lack of fit was detected. The second-order model was fitted with a polynomial model, and the significance of the model was tested using an analysis of variance. The actual and adjusted R-squared values of the model were analyzed. The significance of the model was analyzed by a t-test. The predicted optimum response of the variables was confirmed by running experiments, and enzyme yield was compared. To validate the response, three experiments were performed based on the predicted response. The similarity of the observed and predicted values explains the validity of the designed model.

Table 1. Factors and Levels for Lipase Production in Central Composite Design

Factor	Name	Units	Low Actual	High Actual
A	pH		5.5	7.5
B	Inoculum	%	2	10
C	Moisture	%	40	70

Lipase Compatibility with Detergents

To determine the application of lipase in combination with commercial solid detergents (Rin, Ariel, Surf Excel, Power, Tide, Fino, and Power), they were prepared at 1% concentration (w/v). Then, the detergents were preincubated with the sample for 60 min, and lipase activity was determined.

Analysis of Washing Performance

To determine wash performance, white cotton cloth (3×3 cm^3) was stained with egg yolk and dried at room temperature for 30 min. Then, the stained cloth pieces were immersed in a conical flask containing 50 mL of tap water, detergent, and lipase (200 U/mL). After 30 min, the cloth pieces were removed and rinsed with tap water. Then, the stain-removal properties of lipase were determined.

Lipases in the Treatment of Slaughterhouse Wastewater

The meat processing industries generate significant volumes of wastewater, which includes high organic loads and a significant amount of suspended solids. Slaughterhouse wastewater was collected from a local slaughterhouse involved in goat meat production. In a 250-mL Erlenmeyer flask, 100 mL of slaughterhouse wastewater was added, and 0.5 g, 1g, and 1.5 g of fungal biomass were inoculated. It was maintained in a rotary shaker incubator at 30±1 °C at 150 rpm. After 24 h of treatment, an acetone and ethanol mixture was added to the culture, and the amount of free fatty acid (mM) was evaluated by titrating with 20 mM of sodium hydroxide. Phenolphthalein was used as the indicator. The amount of free fatty acid (mM) was calculated using the following Eq. 2,

$$\text{FFA (mM)} = \frac{(V_a - V_b) * C_{NaOH} * 1000}{m} \quad (2)$$

where V_a is the volume of NaOH (mL) in the control experiment, V_b is the volume of NaOH (mL) in the sample; C_{NaOH} is the molar concentration of NaOH; and *m* is the inoculum load (g).

RESULTS AND DISCUSSION

Screening of Fungi for Lipase Production

The 10 isolated fungi showed lipase activity on polysorbate-80 agar plates. The zone of hydrolysis was 9, 14, 8, 21, 7, 14, 10, 11, 12, and 9 mm, respectively, for the strains LP1, LP2, LP3, LP4, LP5, LP5, LP6, LP7, LP8, LP9, and LP10. In this study, a clear halo was observed around the colony for most of the isolated strains. These fungal strains were further cultured in potato dextrose broth medium, and enzyme activity was assayed. The enzyme activity was assayed from the culture supernatant, and the yields are depicted in Fig. 1.

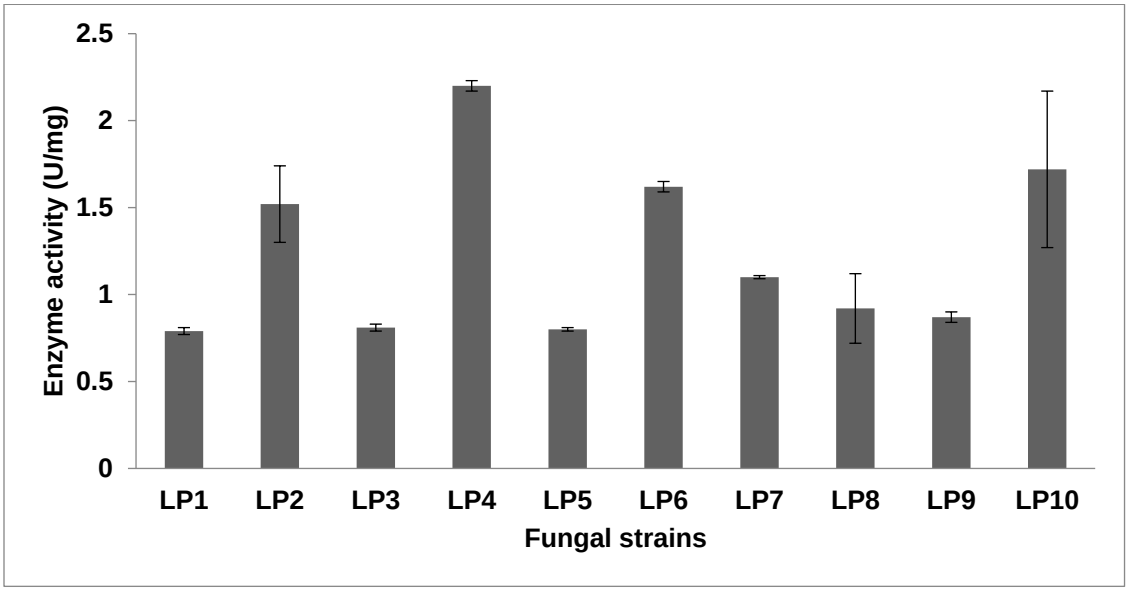

Fig. 1. Lipase activity of fungi isolated from the banana plant vegetation. The morphologically different fungal strains were cultured in potato dextrose broth medium and incubated at 28 ºC for five days. Enzyme activity was expressed ±SD.

Characterization of Fungus

The strain LP4 isolated from the agricultural field exhibited maximum lipase production and was characterized as *Aspergillus niger* LP4. The surface of the fungal colonies was black with conidial production, and the reverse was yellow. Hyphae were septate, and conidial heads split into columns after five days. The growth of the strain was analyzed using PDA medium, and this medium supplied the required nutrients for *A. niger* (Castro Ríos *et al.* 2020). The micro- and macromorphology of *A. niger* varied based on the culture medium and incubation time (Mangal *et al.* 2014).

Oil Cakes on Lipase Production

Three substrates (*C. inophyllum* oil cake, coconut oil cake, and neem seed oil cake) were inoculated with the spore suspensions from *A. niger*LP4 to test the suitable oil cake for the biosynthesis of lipase. *C. inophyllum* oil cake proved to be the best substrate and showed maximum lipase activity (120.2 ± 3.4 U/g) (Fig. 2). The mixture of *C. inophyllum* oil cake and coconut oil cake improved lipase production (Fig. 3). Agricultural residues, such as wheat bran, sugarcane bagasse, and soybean bran, were used as substrates to

produce lipase by *Penicillium* sp. and *Aspergillus* sp. (Fleuri *et al.* 2014). Solid substrates, such as beans, apple pomace, coffee pulp, corn steep dry, sugar cane bagasse, lemon peel, and rice husk, were used for the production of lipase by fungal strains (Kumar and Ray 2014; Sathya *et al.* 2024). Agricultural residues are an important alternative carbon and energy source, playing a significant role in the circular economy (Marraiki *et al.* 2020; Arokiyaraj *et al.* 2024).

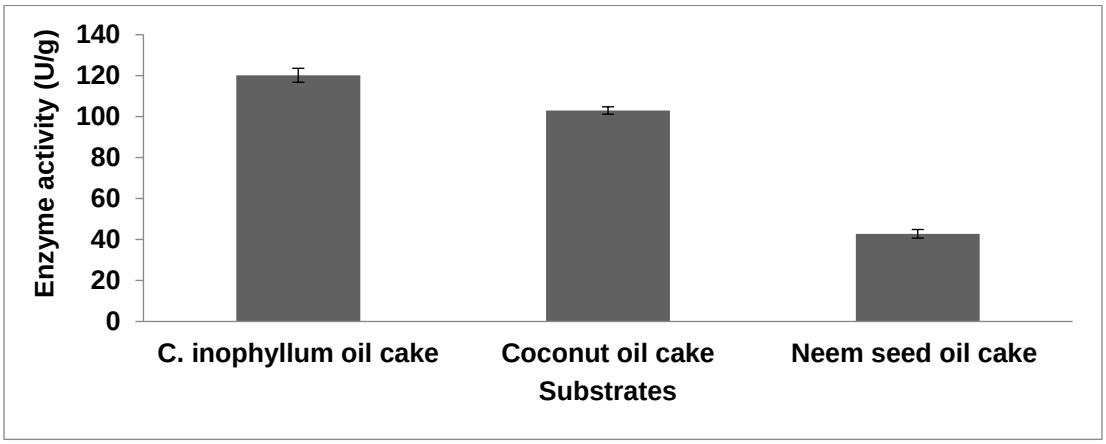

Fig. 2. Effect of oil cakes on lipase production in solid state fermentation. The oil cakes (10 g) were mixed with 0.1 M phosphate buffer and the initial moisture content was 60%. *Aspergillus niger* LP4 was inoculated, incubated at 30 °C for five days, and lipase production was observed.

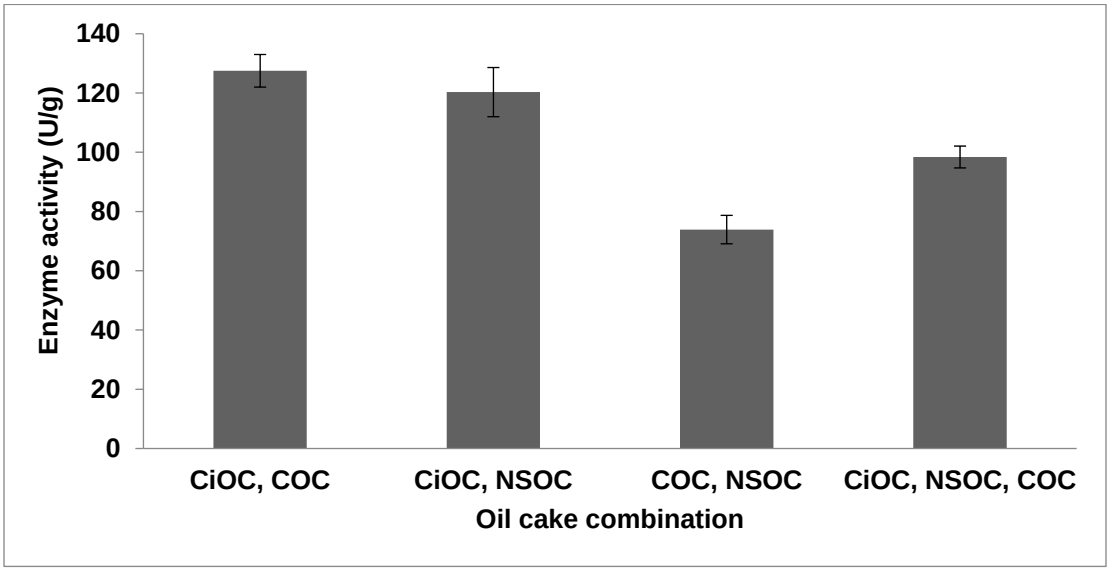

Fig. 3. Screening of combinations of oil cakes for lipase production in solid state fermentation. The combinations were 1:1 (CiOC, COC), 1:1 (CiOC, NSOC), 1:1 (COC, NSOC), and 1:1:1 (CiOC, NSOC, COC). CiOC- *C. inophyllum* oil cake; COC-coconut oil cake; NSOC-neem seed oil cake. *Aspergillus niger* LP4 was inoculated, incubated at 30 °C for five days, and lipase production was observed.

Effect of Temperature

Temperature is an important factor to produce lipase, and different temperature ranges were used. Maximum lipase activity was found at 30 °C in SSF. The variation of lipase yield as a function of temperature was statistically significant ($p<0.01$) (Fig. 4). The significant difference in enzyme yield showed the importance of optimizing temperature

for enzyme production. When the temperature increased above 30 °C or decreased below 30 °C, it decreased lipase production. At lower temperatures, the metabolic activity of fungi was less, and enzyme production decreased. At higher incubation temperatures, enzyme activity was less due to enzyme inactivation (Nema *et al*. 2019).

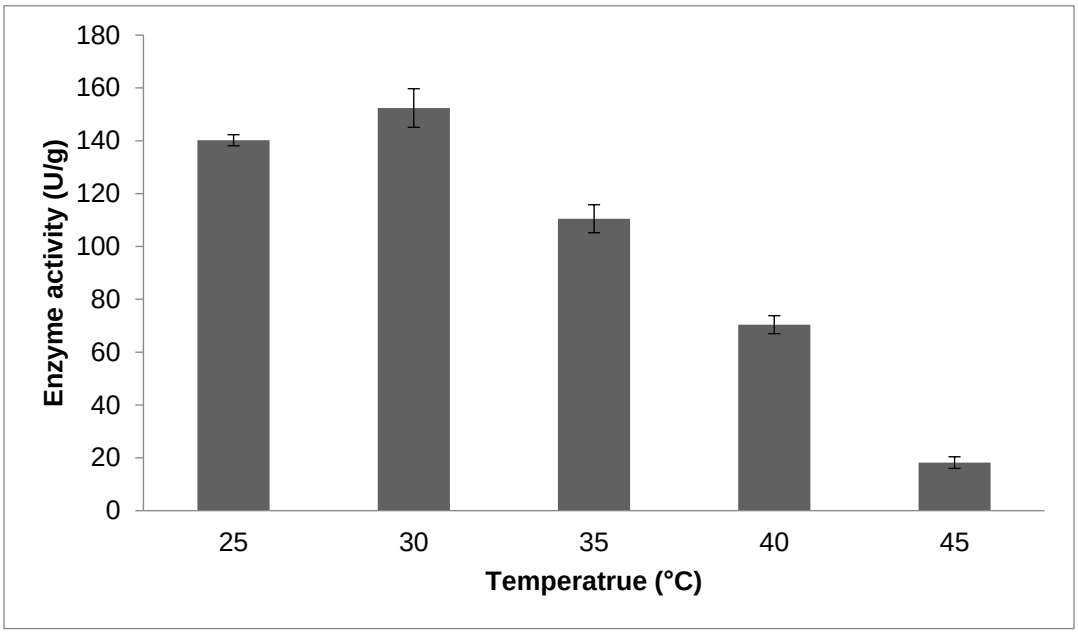

Fig. 4. Effect of temperature on lipase production in solid state fermentation. *Aspergillus niger* LP4 was inoculated in *C. inophyllum* oil cake and coconut oil cake substrate. It was incubated at 30 °C for five days, and lipase production was observed.

pH Optimization of Lipase

To determine the optimum pH for lipase production, buffers with various pH values (5.5 to 7.5) were added.

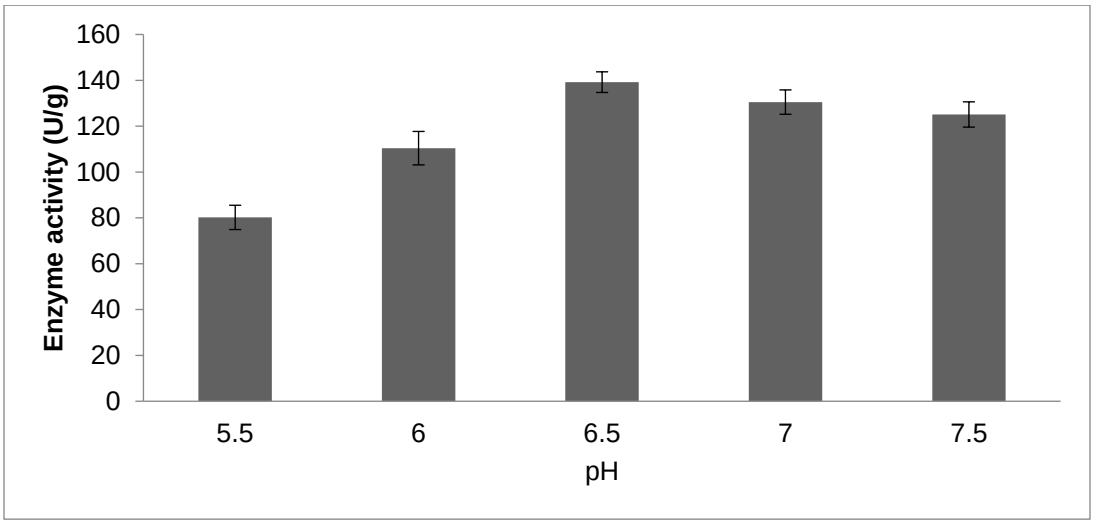

Fig. 5. Effect of pH on lipase production in solid state fermentation. *Aspergillus niger* LP4 was inoculated in *C. inophyllum* oil cake and coconut oil cake substrate. It was incubated at 30 °C for five days, and lipase production was observed.

Lipase production was high at pH 6.5 and it decreased with the lowering of pH values (Fig. 5). The pH of the culture medium is one of the critical factors influencing lipase production in *Aspergillus* sp. The pH value of the substrate influences the transfer of nutritional components, the binding of fungi to the substrate, and the production of final products. The yield of lipase varied based on the type of fungi and fermentation medium selected for solid-state fermentation (Mehta *et al.* 2018).

Inoculum Size Optimization for Lipase Production

The density of spore suspension directly influences fungal growth, colonization, and product formation. In addition, the increased concentration of inoculums reduces the fermentation period and maximizes product formation. In this study, a one-variable-at-a-time approach revealed that 6% inoculum was optimal to produce lipase in solid-state fermentation with *C. inophyllum* oil cake and coconut oil cake substrate. Inoculum size in the 2to4% range and 8 to 10% decreased enzyme yield (Fig. 6). The inoculated spore suspension colonized the solid substrate and penetrated the substrate for improved lipase production. The combined effect of particle size, moisture content, fermentation period, and inoculum size influenced lipase production. Mazhar *et al.* (2017) reported smaller inoculums for optimum lipase production, and the increased inoculum size negatively influenced enzyme production. At higher concentrations of inoculums, the oxygen and nutrient content of the medium becomes depleted before attaining maximum growth of microorganisms (Biji *et al.* 2016; Kalaiyarasi *et al.* 2020).

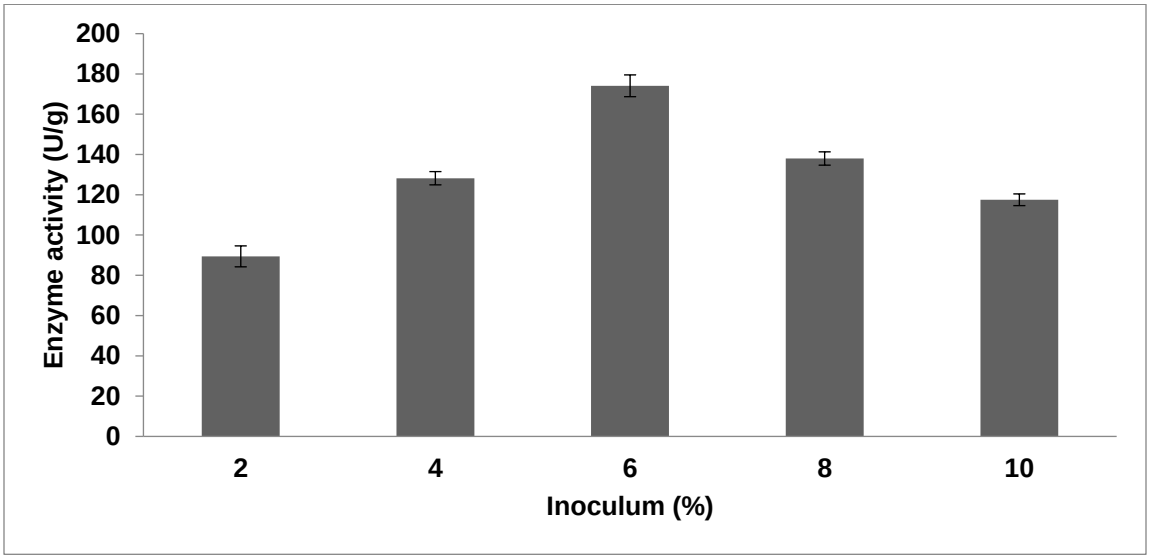

Fig. 6. Effect of inoculums on lipase production in solid state fermentation. *Aspergillus niger* LP4 was inoculated at various percentages (2 to 10%) in *C. inophyllum* oil cake and coconut oil cake substrate. It was incubated at 30 °C for five days, at pH 6.5, and lipase production was observed.

Effect of Moisture on Lipase Production

To determine the effect of moisture content on lipase biosynthesis by *A. niger*, experiments were performed in triplicate for *C. inophyllum* oil cake and coconut oil cake in SSF. Lipase production was maximum at 60% moisture content of the substrate in SSF moisture with phosphate buffer (pH 6.0), as illustrated in Fig. 7. The yield of lipase increased with an increase in the moisture content of the substrate from 40 to 60% (v/w). At higher moisture content (>70%) in the fermented medium, lipase production depleted.

Generally, fungi require lower moisture content for any product formulation than bacteria in SSF. In this study, it was very clear that lipase production was affected by the moisture content of the medium. At low moisture content of the solid substrate, fungal growth was minimal due to the maximum reduction in nutrient diffusion and poor swelling of the substrate material (Baysal *et al.* 2003). In *Aspergillus niger*, 71% moisture content was reported as the optimum moisture content to produce lipase using wheat bran medium (Mahadik *et al.* 2002). The present investigation and previous reports revealed that moisture was a major physical factor determining enzyme yield in SSF.

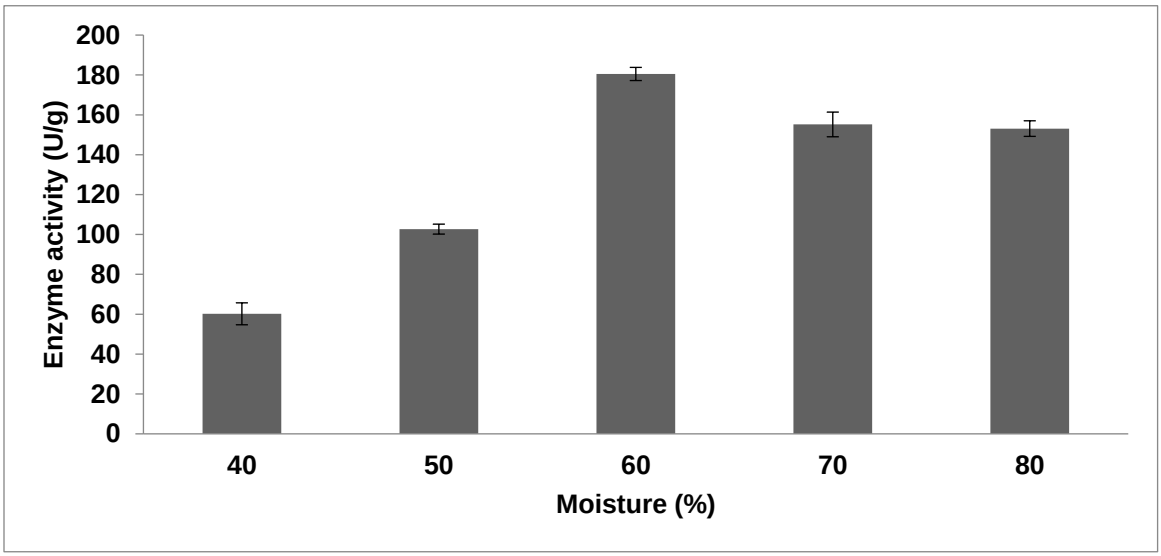

Fig. 7. Effect of moisture content on lipase production in solid state fermentation. *Aspergillus niger* LP4 was inoculated in *C. inophyllum* oil cake and coconut oil cake substrate. It was incubated at 30 °C for five days, and lipase production was observed.

Optimization of Lipase Production Using Response Surface Methodology

After screening bioprocess variables by the traditional method, the physical parameters, such as initial pH, inoculum concentration, and moisture level, were optimized by the central composite design experiment. Response surface models analyze and predict the optimum response based on the interactive effects of variables. Response surface methodology offers a much smaller number of experiments than traditional methods. Response surface analysis for the optimization of variables to obtain the maximum yield (enzyme activity) from *A. niger* LP4 is illustrated in Table 2. Buffers with various pH values were used to maintain the moisture content of the solid substrate. *C. inophyllum* oil cake and coconut oil cake favored the production of lipase in SSF at acidic pH values. Optimization of culture medium pH is one of the important factors because pH directly affects the physiological and metabolic processes of fungi. The experiment was modeled with response surface methodology, and statistical analysis showed that RSM can be used to predict lipase yield. The predicted R-squared value of the model was 0.9205, and the adjusted R-squared value was 0.8489. The model F-value of 12.98 implies that the model was significant (Table 3). In this CCD model, A, B, C, A^2, and C^2were significant model terms.

Final Equation in Terms of Coded Factors:

$$\text{Lipase activity} = +338.32 + 142.30A + 38.68B + 46.98C + 26.01AB - 14.76AC - 33.29BC - 48.93A^2 - 30.24B^2 - 38.82C^2 \quad (3)$$

Table 2. Production of Lipase Using Central Composite Design

Runs	A-pH	B-Inoculum	C-Moisture	Lipase (U/g)
1	5.5	2	70	98.3
2	5.5	2	40	17
3	6.5	6	55	349.3
4	5.5	10	70	98.3
5	6.5	-0.72	55	164.2
6	6.5	6	29.77	97.4
7	7.5	10	40	487.2
8	6.5	6	55	265
9	6.5	6	80.22	364.3
10	4.81	6	55	3.2
11	6.5	12.72	55	346
12	7.5	2	70	398.2
13	6.5	6	55	339
14	6.5	6	55	387.2
15	6.5	6	55	360.3
16	7.5	10	70	376.3
17	7.5	2	40	250
18	6.5	6	55	328.3
19	5.5	10	40	24.2
20	8.18	6	55	401.3

Table 3. Analysis of Variance for Central Composite Design.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4.02E+05	9	44637.96	12.98	0.0002
A-pH	2.77E+05	1	2.77E+05	80.41	< 0.0001
B-Inoculum	20432.82	1	20432.82	5.94	0.035
C-Moisture	30139.64	1	30139.64	8.76	0.0143
AB	5413.2	1	5413.2	1.57	0.2382
AC	1743.45	1	1743.45	0.51	0.4928
BC	8864.46	1	8864.46	2.58	0.1395
A^2	34499.58	1	34499.58	10.03	0.01
B^2	13180.68	1	13180.68	3.83	0.0787
C^2	21713.4	1	21713.4	6.31	0.0308
Residual	34394.4	10	3439.44		
Lack of Fit	25924.89	5	5184.98	3.06	0.1225
Pure Error	8469.51	5	1693.9		
Cor Total	4.36E+05	19			

The main effect of the selected variables and their interactions on the yield can be analyzed using central composite models and 3D response surface graphs (Bagewadi *et al.* 2018). The 3D response surface plot shows the optimum concentration of factors (two factors), whereas the third variable was at the middle level. The elliptical 3D-response surface graph revealed the good interaction of selected factors for SSF (Hasan *et al.* 2023). To improve the maximum lipase yield, a 3D plot was constructed between the inoculum and moisture content. The response surface of pH *versus* moisture content revealed that the production of lipase was improved when the pH of the medium was maintained between 6.0 and 6.5.

The response surface plot of the variables is described in Fig. 8. To improve maximum lipase production, a response surface graph was obtained, and the yield analyzed. This 3D plot revealed improved lipase activity with increased moisture content in the medium. A 3D response surface plot between inoculums and pH was also analyzed in this study, and enzyme activity was presented on the z-axis. The interactions are represented in Fig. 8a.

Figure 8b represents the interactive effect of moisture and pH. At low pH and moisture content, lipase production was very low, and peak production was achieved at higher pH (6to6.5) and moisture content. When compared with moisture content, the pH of the culture medium had a significant effect on enzyme production (Fig. 8b). The interactive effect of moisture and inoculum is shown in Fig. 8c. The results from the 3D response surface plots showed that moisture had a greater effect than inoculum concentration. Mhetras *et al.* (2009) reported the significance of pH on lipase production by *Aspergillus niger* NCIM 1207. In this work, enzyme production improved 3-fold after statistical optimization than unoptimized medium in solid state fermentation. The statistical methods can be used on a commercial scale for industrial production of biomolecules, including enzymes (Saeed *et al.* 2023; Wahab *et al*. 2023; Zhang and Wu 2023).

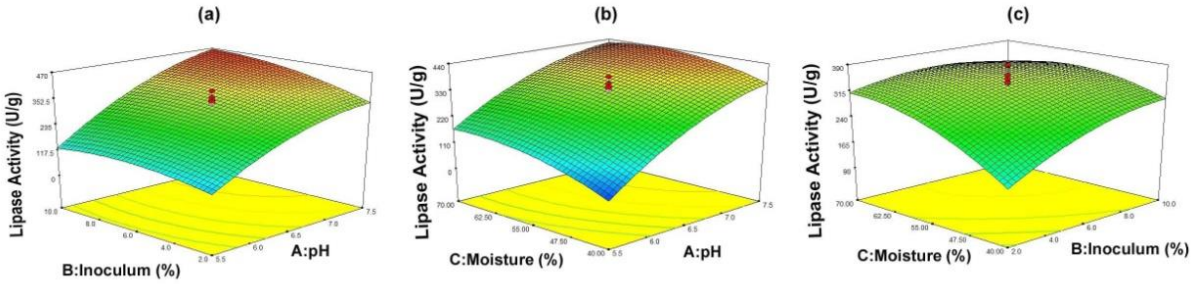

Fig. 8. Response surface Graph for the production of lipase in solid state fermentation. (a) Interactive effect of inoculum and pH, (b) Moisture and pH, and (c) Moisture and inoculum

Lipase Compatibility with Detergents

The results showed excellent stability and compatibility with solid detergents such as Rin, Ariel, Surf Excel, Power, Tide, and Fino (Fig. 9). Lipase activity was retained, and 100% relative activity was observed in this study. The present finding revealed the compatibility and stability of lipase extracted from a fungal strain.

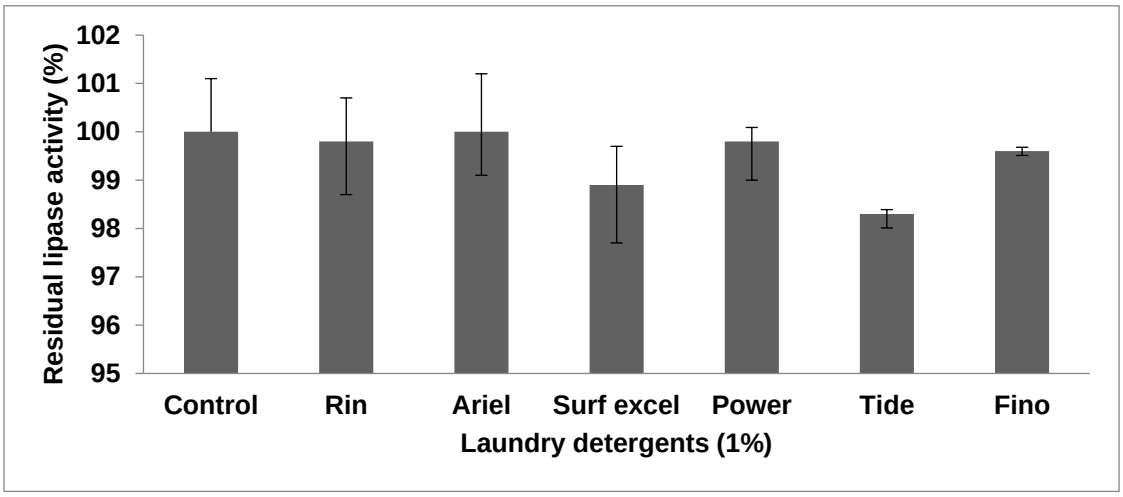

Fig. 9. Compatibility of fungal lipase in the presence of commercial detergents. Fungal lipase was incubated with detergents (1 g/100 mL) and incubated for 60 min. Detergent was not incorporated in the control. After 60 min of preincubation, the remaining lipase activity was analyzed.

Wash Performance Analysis

Toanalyze the suitability of lipase for stain removal, an experiment was conducted to remove egg yolk stain from cotton cloth (Fig. 10). The egg yolk removal percentage was higher in the egg yolk-stained cloth treated with enzymes and detergent. The efficacy was higher than that of stained cloth treated with detergent alone. This implies the potential of lipase in stain removal.

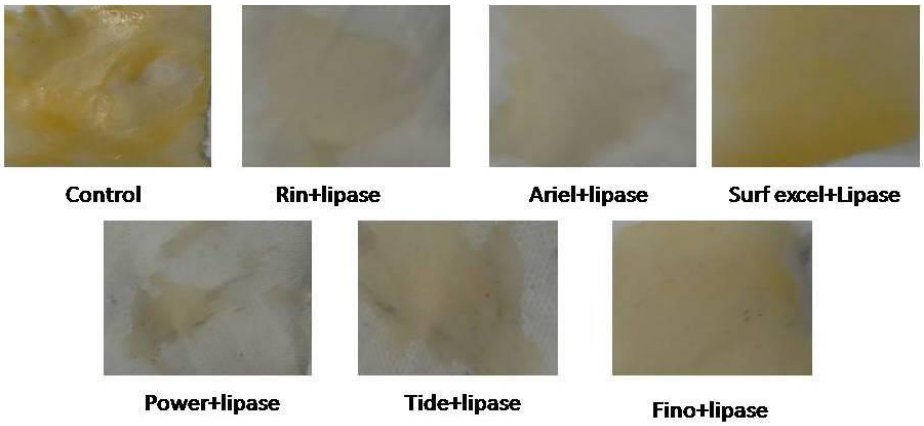

Fig. 10. Wash performance test on egg yolk removal with detergent in the presence of lipase. The stained cotton cloth was incubated in detergent and lipase (200 U/mL). After 30 min, the cloth pieces were removed, rinsed, and the stain-removal properties of lipase were determined.

Free Fatty Acid Generation from the Slaughterhouse Wastewater by Lipase Producing Fungus

To determine the influence of fungal lipases on free fatty acid generation from the slaughterhouse wastewater, fungal biomass was added to the slaughterhouse waste, and the amount of free fatty acid generation was determined. The result is depicted in Fig. 11.At 0.5 g of biomass, the generation of fatty acids was less, and it was improved with increasing concentrations of fungal biomass. Lipase-producing microorganisms are involved in the hydrolysis of organic matter and the liberation of free fatty acids in the effluent (Duarte *et*

al. 2015; Qiao *et al.* 2017). Valladão *et al.* (2007) used enzymes for the pretreatment of solid wastes. The solid enzymatic pool improved the formation of fatty acid and removed organic matter from the effluent.

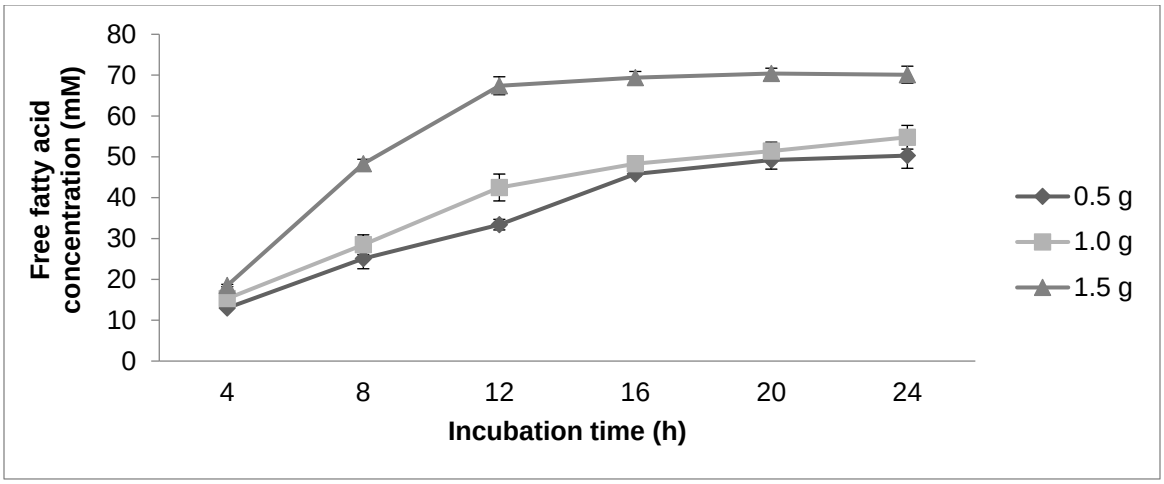

Fig. 11. Effect of fungal biomass on the formation of free fatty acids from the hydrolysis of slaughterhouse wastewater

CONCLUSIONS

1. *Calophyllum inophyllum* oilcake and coconut oil cake mixtures were used as low-cost substrates to produce lipase in solid-state fermentation.
2. The low-cost lignocellulosic biomass reduced the use of costly synthetic mediums.
3. The utilization of solid mediums facilitates the utilization of oily substrates and the reduction of environmental pollution.
4. The combination of *Calophyllum inophyllum* oilcake and coconut oil cake waste improved lipase production in *Aspergillus niger* LP4.
5. The crude lipase improved detergent compatibility, hydrolyzed biomass from the slaughterhouse wastewater, and released free fatty acids.

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