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Lipase Biosynthesis by Aspergillus niger Utilizing Solid Waste as Feedstock: Advancing a Circular Bioeconomy Approach

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ABSTRACT

Lipase production was carried out using agro-industrial residues mustard oil cake (MC), wheat straw (WS), and olive oil (OO) as substrates in submerged fermentation by *Aspergillus niger*. Two concentrations (1.2% and 2.4% w/v) were tested for each substrate. The highest enzyme activity was observed with mustard oil cake at 2.4% (MC2), reaching 480 U/mL after 72 hours of incubation, followed by 450 U/mL for olive oil at 2.4% (OO2) and 360 U/mL for wheat straw at 2.4% (WS2). A continuous decrease in glucose concentration confirmed substrate utilization, with maximum consumption in MC1. Protein concentration peaked at 8.1 mg/mL in MC2 at 72 h, suggesting efficient enzyme synthesis. Purification of the enzyme through ammonium sulfate precipitation (80% w/v) followed by ion exchange chromatography increased the specific activity significantly, reaching 415.14 U/mg for MC2. The percentage yield after salting-out was highest for MC2 (361.11%), indicating the effectiveness of this substrate. Overall, mustard oil cake outperformed wheat straw and olive oil in terms of lipase yield, purification efficiency, and substrate utilization. These results demonstrate the potential of low-cost solid waste substrates in cost-effective enzyme production aligned with circular bioeconomy principles.

Keywords: *Lipase, Aspergillus niger, Submerged fermentation, Purification, Enzyme*

1. INTRODUCTION

Lipases have garnered increasing global attention due to their broad industrial relevance and remarkable catalytic versatility. These enzymes play a critical role in biocatalysis and are extensively used in sectors such as biofuel production, food processing, cosmetics, pharmaceuticals, and organic synthesis [1]. Lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) catalyze the hydrolysis of triglycerides into glycerol and free fatty acids at oil–water interfaces [2], with a unique ability to function in non-aqueous media, enabling the synthesis of esters, alcohols, and other value-added products [3]. Globally, lipases account for nearly 75% of all industrial enzyme applications, with Europe leading as a major producer [4].

Among various sources, microbial lipases especially from fungi have become the preferred choice due to their extracellular secretion, high yield, and stability under industrial conditions [5]. Fungi like *Aspergillus, Rhizopus, Mucor*, and *Candida* species are known lipase producers, commonly isolated from environments such as oil-contaminated soils, compost heaps, and industrial effluents [6]. These microbial lipases have been extensively studied and characterized for their kinetic properties, stability, and tolerance to pH, temperature, and metal ions [7]. Several purification techniques such as salting out, ion exchange chromatography, and gel filtration are commonly employed to obtain purified enzyme fractions [8].

While solid-state fermentation (SSF) is traditionally preferred for fungal lipase production, submerged fermentation (SmF) offers better control over process parameters, homogeneous nutrient distribution, and scalability for industrial operations [9].

In this study, we investigated lipase biosynthesis by *Aspergillus niger* using agro-industrial solid wastes mustard oil cake and wheat straw as feedstock substrates under submerged fermentation.

Both are abundant, low-cost by-products of the oil extraction and agricultural industry, respectively, aligning with the principles of circular bioeconomy. A kinetic study was also performed to evaluate substrate utilization, protein production, and enzyme activity. The resulting enzyme was purified through ammonium sulfate precipitation and ion exchange chromatography.

2. MATERIALS AND METHODS

The filamentous fungus *Aspergillus niger* previously isolated and maintained in our laboratory was used for lipase production. The culture was preserved on potato dextrose agar (PDA) slants containing 200 g/L potato infusion, 20 g/L dextrose, and 20 g/L agar, and incubated at 28°C for 96 hours.

2.1 Inoculum Preparation

For inoculum preparation, a loopful of fungal culture was transferred aseptically into 10 mL of sterilized potato dextrose broth (PDB) prepared with 200 g/L potato and 20 g/L dextrose. The culture was incubated at 28°C for 72 hours under static conditions.

2.2 Pretreatment of Substrates

Mustard oil cake and wheat straw were selected as solid waste substrates for lipase production, along with olive oil as a control. Mustard oil cake was pretreated using distilled water to moisten and soften the matrix, while wheat straw was subjected to acid hydrolysis using 0.1% (v/v) sulfuric acid to break down lignin and release fermentable sugars for microbial utilization.

2.3 Fermentation Media and Conditions

The production medium used for submerged fermentation consisted of (in g/L): glucose 12.5, yeast extract 0.25, peptone 0.25, KH_2PO_4 2.0, $MgSO_4 \cdot 7H_2O$ 0.5, and KCl 0.5. The media was sterilized by autoclaving at 121°C for 15 minutes. Fermentation was conducted in 250 mL Erlenmeyer flasks containing 100 mL of sterile production media supplemented with either 1.2% w/v or 2.4% w/v of mustard oil cake (MC1 & MC2), wheat straw (WS1 & WS2), or olive oil (OO1 & OO2). The inoculum was then added to each flask, and cultures were incubated at 28°C in an orbital shaker at 120 rpm for 96 hours.

2.4 Estimation of Protein and Sugar

Total protein concentration was determined using the Lowry method, whereas reducing sugar concentration was estimated using the dinitrosalicylic acid (DNS) method, following standard protocols [10].

2.5 Lipase Activity Assay

Lipase activity was measured using olive oil as the substrate. The reaction mixture included 3.0 mL of olive oil, 2.5 mL of deionized water, 1.0 mL of 200 mM Tris-HCl buffer (pH 7.5), and 1.0 mL of enzyme extract. The mixture was incubated at 37°C for 30 minutes. After incubation, 3.0 mL of ethanol and a few drops of phenolphthalein were added. The mixture was titrated against 50 mM NaOH, with the endpoint marked by a persistent pink color. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions [11].

2.6 Crude Enzyme Isolation

After 96 hours of fermentation, the broth was filtered through muslin cloth to remove biomass. The filtrate was then centrifuged at 5000 rpm for 15 minutes at 4°C. The resulting supernatant was collected and used as crude enzyme extract for subsequent analysis of protein, sugar, and lipase activity.

2.7 Enzyme Precipitation by Salting Out

Saturated ammonium sulfate was gradually added to the crude enzyme extract to achieve 80% saturation. The solution was kept at 4°C and centrifuged at 5000 rpm for 20 minutes. Pellet formation was monitored after each increment of ammonium sulfate (in 2 mL steps). The final protein pellet obtained was dissolved in phosphate buffer and stored at 4°C for further purification and analysis [12].

3. RESULTS AND DISCUSSIONS

Lipase was produced using mustard oil cakes, wheat straw, and olive oil as substrates in the submerged fermentation. The kinetics study for lipase production was done for the estimation of the product yield and substrate utilization. The kinetic study was carried out for 96h at the intervals of 0h, 24h, 48h, 72h, and 96h on all the sets of experiments carried out on all the substrates. The concentration of substrate selected was 1.2% w/v and 2.4% w/v in the case of all the substrates, such as mustard oil cakes, wheat straw, and olive oil, denoted as MC1 & MC2, WS1 & WS2, and OO1 & OO2, respectively.

3.1 Kinetics of Lipase Production and Substrate Utilization

The kinetics of lipase production were investigated using *Aspergillus niger* in submerged fermentation at various intervals (0, 24, 48, 72, and 96 hours) employing different substrates at 1.2% and 2.4% concentrations. The substrates used were mustard oil cake (MC1 and MC2), wheat straw (WS1 and WS2), and olive oil (OO1 and OO2). Maximum enzyme activity was observed at 72 hours across all treatments, indicating this as the optimal time point for lipase production. Among all, MC2 exhibited the highest activity (480 U/mL), followed by MC1 (440 U/mL), OO2 (450 U/mL), OO1 (380 U/mL), WS2 (360 U/mL), and WS1 (310 U/mL). A subsequent decline in activity at 96 hours was noted, possibly due to enzyme denaturation or nutrient depletion. These findings suggest that higher concentrations of mustard oil cake and olive oil enhance lipase production, with mustard oil cake being the most effective substrate under the given conditions (Figure 1).

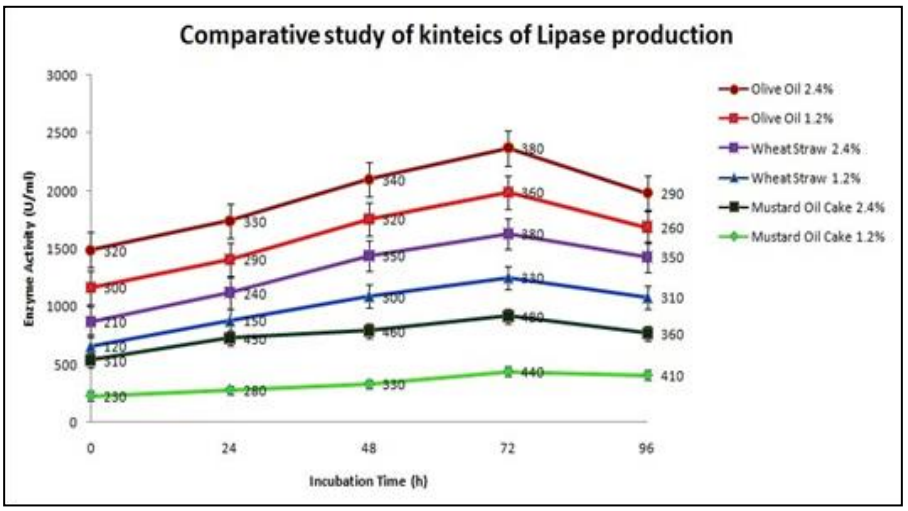

Figure 1. Comparative study of kinetics of lipase production.

3.2 Glucose Utilization

Glucose consumption was analyzed to assess metabolic activity during fermentation. A consistent decrease in glucose concentration was recorded in all treatments over the 96 hours, confirming active metabolism by *A. niger* (Figure 2). The most substantial reduction was observed in MC1, where glucose declined from 9.8 mg/mL to approximately 2.5 mg/mL. This indicates efficient carbon source utilization, particularly when mustard oil cake was used. Such utilization patterns correlate with elevated enzyme synthesis, affirming that glucose was effectively channeled towards lipase biosynthesis in these cultures.

The study of the kinetics of the enzyme for glucose content shows that glucose has decreased with the increasing number of days, showing that the lipase enzyme has utilized the carbohydrate source for performing its activities.

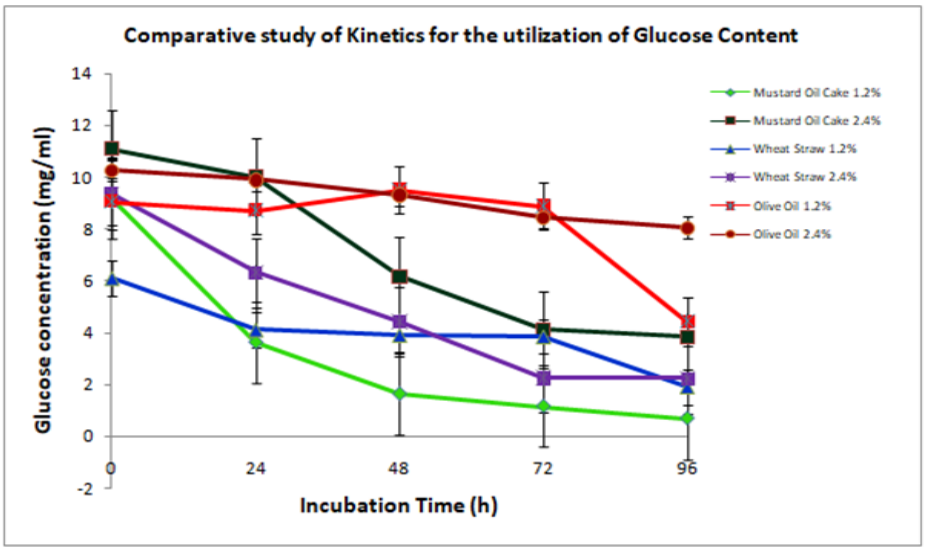

Figure 2. Comparative study of kinetics for the utilization of glucose content.

3.3 Kinetics study for protein content produced in various lipase production processes

Total protein content was found to increase progressively until 72 hours, after which a decline was observed, suggesting peak enzyme synthesis at this time point. This trend may be attributed to the exhaustion of nutrients or degradation of synthesized proteins beyond 72 hours (Figure 3). The highest protein concentration was recorded in MC2 (8.10 mg/mL), followed by MC1 (6.86 mg/mL), WS2 (5.0 mg/mL), and WS1 (4.2 mg/mL). In contrast, olive oil samples (OO1 and OO2) displayed minimal protein production (<2.0 mg/mL). These results indicate that mustard oil cake not only supports higher lipase activity but also promotes increased protein synthesis, underscoring its potential as a preferred substrate for fungal fermentation.

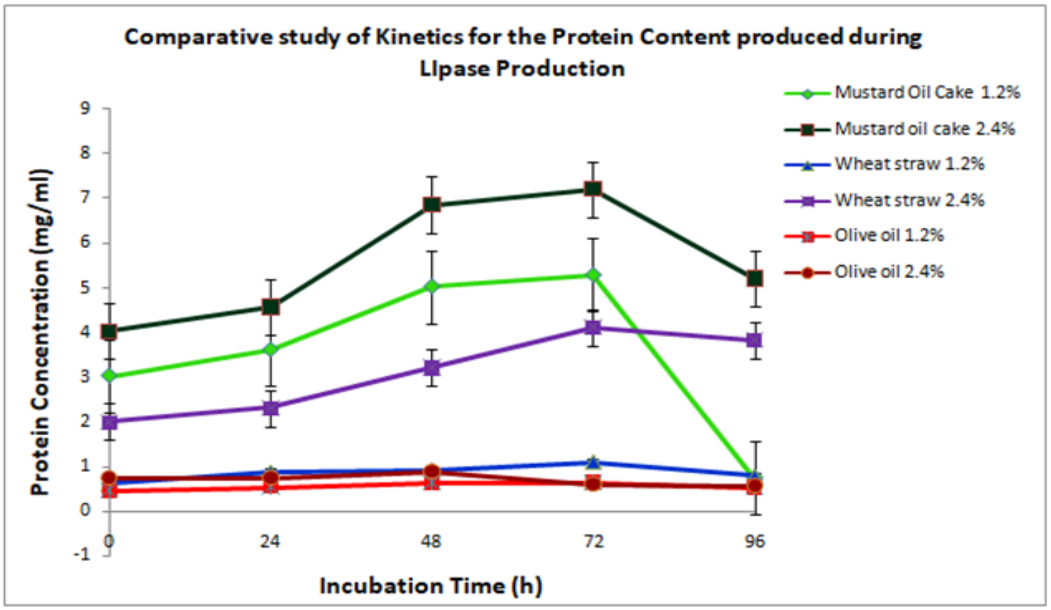

Figure 3. Comparative study of kinetics for the production of protein content.

3.4 Kinetic studies for partial purification of lipase enzyme

Purification of the crude enzyme extracts was carried out via ammonium sulfate precipitation (80%) followed by centrifugation. A marked improvement in specific activity was recorded post-purification. Notably, the MC2 sample exhibited a specific activity of 415.14 U/mg with a yield of 361.11%, demonstrating its effectiveness in supporting high-yield lipase production. Remarkably, WS1 exhibited the highest specific activity post-salting (5128.68 U/mg), despite having a relatively lower crude enzyme activity. Purification folds varied across treatments, with significant increases observed in MC2 (3.48 to 6.01) and WS1 (3.93 to 13.02), while moderate enhancements were seen in WS2 (0.91 to 1.84) and MC1 (0.40 to 0.50). Interestingly, olive oil treatments (OO1 and OO2) showed a reduction in purification fold (3.33 to 0.53 and 3.14 to 0.77, respectively), indicating that the precipitated proteins may not represent highly active lipase or could contain interfering impurities. These observations highlight the suitability of mustard oil cake and wheat straw as efficient substrates for lipase recovery and purification (Table 1).

Table 1. Protein content, enzyme activity, specific enzyme activity and %yield after different purification steps in mustard oil cake, wheat straw and olive oil samples.

Purification Steps	Protein Content (mg/mL)	Enzyme Activity (U/mL)	Specific Enzyme Activity (U/mg)	% Yield
Mustard Oil Cake 1.2%w/v				
Fermented Broth after 96 hrs incubation	0.7540	410	543.7540	_
After removal of insoluble	1.7923	390	217.5931	95.1219

Salting out by Ammonium Sulphate	2.4103	650	269.6666	158.5366
Mustard Oil Cake 2.4%w/v				
Fermented Broth after 96 hrs incubation	5.2121	360	69.0687	_
After removal of insoluble	2.3279	560	240.5522	155.5556
Salting out by ammonium Sulphate	3.1314	1300	415.1447	361.1111
Wheat Straw 1.2%w/v				
Fermented Broth after 96 hrs incubation	0.7871	310	393.8523	_
After removal of insoluble	0.6967	340	487.9630	109.6774
Salting out by ammonium Sulphate	2.3485	450	5128.6800	132.3529
Wheat Straw 2.4%w/v				
Fermented Broth after 96 hrs incubation	3.8256	350	91.4886	_
After removal of insoluble	1.2673	390	307.7384	111.4286
Salting out by ammonium Sulphate	2.8562	480	168.0537	123.0769
Olive Oil 1.2% w/v				
Fermented Broth after 96 hrs incubation	0.5768	260	450.7286	_
After removal of insoluble	0.1977	280	1415.7500	107.6923
Salting out by ammonium Sulphate	1.2937	450	347.8185	173.0769
Olive Oil 2.4% w/v				
Fermented Broth after 96 hrs incubation	0.5480	290	529.1955	_
After removal of insoluble	0.2101	370	1760.7650	127.5862
Salting out by ammonium Sulphate	2.1260	600	282.2093	206.8966

4. CONCLUSION

The study demonstrates that *Aspergillus niger* efficiently produces lipase using agro-waste substrates under submerged fermentation. Among the substrates, mustard oil cake at 2.4% concentration (MC2) yielded the highest enzyme activity (480 U/mL) and protein content (8.1 mg/mL) at 72 h. Glucose consumption patterns confirm active substrate utilization, especially

in MC1. Post-purification analysis showed that MC2 also achieved the best recovery and specific activity, confirming it as the most suitable substrate. Wheat straw showed moderate efficiency, while olive oil performed the least effectively in terms of fold purification and protein concentration. This study validates the use of agro-industrial residues, particularly mustard oil cake, as cost-effective feedstocks for lipase production in a circular bioeconomy framework.

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6. AUTHOR CONTRIBUTION

Conceptualization, M.N., G.A.; methodology, M.N., V.S. and S.D.; formal analysis, V.G., T.T.; investigation, G.A.; data curation, V.S., T.T.; writing original draft preparation, M.N.; writing, review and editing, V.S. V.G. S.D.; supervision, G.A.; All authors have reviewed and approved the final version of the manuscript for publication.

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