FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 9 April Special Issue, 2025, pp 79 - 83
DOI: https://doi.org/10.33003/fjs-2025-09(AHBSI)-3498

OPTIMIZATION OF LIPASE PRODUCTION USING AGRO-ALLIED WASTES BY *Bacillus subterraneous* IN SOLID-STATE FERMENTATION

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ABSTRACT

Lipases are industrially significant enzymes widely used in food, detergent, pharmaceutical, and biodiesel industries. This study investigates the optimization of lipase production by *Bacillus subterraneous* isolated from abattoir soil in Kurmin mashi Abattoir, Kaduna using agro-allied wastes as substrates in solid-state fermentation (SSF). Soil samples were collected and analyzed to isolate lipase-producing bacteria, which were identified through morphological, biochemical, and molecular techniques. Various agro-wastes such as wheat bran, rice bran, sugarcane peel, and groundnut husk were evaluated for their efficiency in supporting microbial growth and enzyme production. The study aimed to enhance enzyme yield by optimizing key fermentation parameters, including pH, temperature, substrate concentration, incubation time, and agitation speed. The highest lipase activity of 6.90±0.10 U/mL was achieved under optimal conditions: pH 6, temperature 40°C, inoculums level 4.0% (w/v), and incubation time 48 hours. Among the tested agro-allied wastes, groundnut shell and groundnut oil proved to be the most effective substrate for lipase induction. The enzyme was subsequently purified using ammonium sulfate precipitation, dialysis, and gel filtration chromatography. SDS-PAGE analysis confirmed the molecular weight of the purified lipase to be 25kDa. The findings suggest significant industrial potential, particularly in biodegradable detergents, biodiesel production, food processing, and pharmaceutical applications. Moreover, the utilization of agro-waste as a fermentation substrate highlights the economic and environmental sustainability of this production approach, supporting waste valorization and circular bioeconomy models.

Keywords: Lipase, *Bacillus subterraneus*, Solid-State Fermentation, Agro-Allied Wastes, Enzyme Optimization

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze the hydrolysis of fats into glycerol and free fatty acids. These enzymes have broad industrial applications, including their use in food processing, detergents, pharmaceuticals, and biodiesel production (Hasan*et al.*, 2006). Their ability to catalyze a wide range of bioconversion reactions, such as esterification, interesterification, and transesterification, has made them a focus of biotechnological research (Ghosh *et al.*, 2016). However, large-scale enzyme production is often limited by high production costs, necessitating the exploration of low-cost and sustainable substrates for microbial fermentation. Agro-allied wastes, such as wheat bran, rice bran, coconut husk, and groundnut shell, are potential sources of carbon and nutrients that can support microbial growth and enzyme synthesis (Ghosh *et al.*, 2016).

Microbial lipases are preferred over plant and animal-derived lipases due to their stability, ease of production, and ability to function under diverse conditions. Solid-state fermentation (SSF) has gained attention as a favorable technique for microbial enzyme production due to its economic and environmental benefits. SSF involves the growth of microorganisms on solid substrates with minimal free water, which closely mimics their natural habitat (Pandey *et al.*, 1999). Compared to submerged fermentation (SmF), SSF offers advantages such as higher enzyme yields, reduced contamination risks, lower water consumption, and simpler downstream processing. Additionally, SSF allows for the effective utilization of agricultural residues, contributing to waste valorization and sustainable bioprocessing (Singhania *et al.*, 2009).

Bacillus subterraneous has emerged as a promising producer of lipases, owing to its robust enzymatic activity, rapid growth, and capability to utilize a variety of carbon sources. Several studies have demonstrated that members of the *Bacillus* genus are highly efficient lipase producers, making them ideal candidates for industrial applications (Rathi*et al.*, 2001). In a research previously published by this authors (Magashi *et al.*, 2025), *Bacillus subterraneous* was isolated from soil of abattoir and Identified using both phenotypic and molecular identification techniques. The isolates was further screened for lipase production and was revealed to be a good lipase producer compared to other bacteria screened. This study aims to optimize lipase production by *Bacillus subterraneous* using agro-allied wastes as substrates in SSF. The specific objectives include evaluating different agro-waste materials for their effectiveness in supporting microbial lipase production, optimizing fermentation parameters such as moisture content, pH, temperature, and incubation time, and assessing the potential industrial applications of the produced enzyme. The findings from this study will contribute to the development of an eco-friendly and cost-effective strategy for potential large-scale lipase production.

MATERIALS AND METHODS

Selection of Agro-Allied Wastes

The selection of agro-allied wastes was based on their availability, nutrient composition, and previous reports on their effectiveness in microbial enzyme production. The selected substrates were:Wheat bran, Rice bran, Brown

Sugarcane peel, Groundnut shell, Each agro-waste was dried at 60°C for 24 hours, ground into fine particles (where applicable), and stored in airtight containers until use.

Solid-State Fermentation (SSF)

The SSF experiments was carried out in 250 ml Erlenmeyer flasks containing 10g of each agro-waste substrate, 1.0 ml of micronutrient solution having composition of 5.0 g/L K_2HPO_4, 1.0 g/L $MgSO_4.7H_2O$, 1.98 g/L $MnCl_2.4H_2O$, 0.25 g/L $CuSO_4.5H_2O$ and 103 0.29 g/L $ZnSO_4.7H_2O$ with pH 7.0, the final substrate moisture content was adjusted to 50% (v/w) using distilled water. After sterilization by autoclaving, the flasks were cooled and inoculated with 1.0 ml of inoculum (1.5×10^8cfu/mL) and 1mL of each of Groundnut oil, olive oil and coconut oil each and incubated at 37°C for 48 hours. The flasks were incubated at different temperatures, pH levels, and moisture conditions to optimize lipase production. The supernatant was analyzed for lipase activity using spectrophotometric method (Saadatullah *et al.,* 2018).

Optimization Parameters

To determine the optimal conditions for lipase production, different physicochemical parameters were varied systematically:

Moisture Content: Adjusted to 30%, 40%, 50%, 60% and 70%, using sterile distilled water.

Incubation Period: Lipase activity was monitored at 24hrs, 48hrs, 72hrs and 96hrs intervals

pH Variation: The initial pH of the medium was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 using 0.1N HCl or NaOH.

Temperature: Flasks were incubated at 30°C, 40°C, 50ºC, 60ºC and 70°C.

Carbon and Nitrogen Sources: Various carbon sources (glucose, sucrose, lactose, fructose and maltose) and nitrogen sources (yeast extract, malt extract and urea) were added to the fermentation medium to assess their effects on lipase production.

Lipase Assay

The substrate solution was prepared by adding 1ml of solution A and 19ml of solution B, the assay mixture contained 1ml of the substrate, 0.5ml of buffer, 0.1ml of enzyme and final volume made up to 3ml with distilled water. The enzyme activity was stopped by adding 0.2ml isopropanol and liberation of P-nitrophenol at 45^0C detected in spectrophotometer at 410nm. One enzyme is defined as 1 mol of P-nitrophenol enzymatically released from the substrate per minutes (Syed *et al.,* 2010).

OD_{410}=5.43020 x Conc[mM]+0.00303

Conc(mM) of p-nitrophenol released =

$$\frac{OD_{410}-0.00303}{5.43020} = A$$

Enzymes (uml^{-1}) A x 20

Determination of Molecular weight of Lipase

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) Using Express Plus TM PAGE Gels

Preparation of Gel Buffer and Gel Tank

A commercial kit containing a pack of MOPS running buffer powder (Cat No. M00138) was dissolved in 1 L deionized water to make 1 L 1x running buffer. Express Plus^{TM}PAGE Gel from the pouch was removed and the sealing tape at the bottom of the gel cassette was peeled. The comb was removed from the gel cassette gently and inserted into the gel running apparatus and sufficient 1x MOPS running buffer was poured into the inner tank of the gel running apparatus to cover the sample wells by 5-7 mm while the outer tank was filled with 1x MOPS running buffer to ensure proper cooling. The buffer in the outer tank was above the top level of the sample wells, this was rinsed thoroughly with 1x running buffer to remove air bubbles and displace any storage buffer. The 4x protein sample buffer, 8ul and 32ul of sample was mixed and heated at 95ºC for 5mins.

The samples as well as protein standard were loaded directly into the wells, the tank was closed and ran at 140volt for 1hour, after which the gel was carefully removed from the Cassette. Once the run was completed, the gel was removed from the gel tank according to the manufacturer's instructions. The gel cassette was opened carefully by inserting the cassette opener into the gap between the two plates. The cassette-opener was wiggled up and down gently to separate the two plates and repeated along both sides of the cassette, until the two plates are completely separated with a cracking sound which indicates the opening of the cassette. Upon opening, the gel was removed and the plate discarded without the gel, the gel was allowed to stay on the plate which was loosened from the plate and removed carefully.

Staining and Drying of Gels

The staining procedures provided with commercial kit;Express Plus ^{TM} PAGE gels was prepared according to manufacturer's guide. The staining solution was mixed with 0.1% Coomassie R-250, dissolved in a 40% ethanol, 10% acetic acid solution. The destaining solution was mixed with 10% ethanol, 7.5% acetic acid solution according to manufacturer's guide.

The gel cassette was carefully opened and taken out after electrophoresis; the gel was placed in a staining container with 100ml staining solution, covered and heated in a microwave oven at full power for 8 minutes without allowing the solution to boil according to manufacturer's warning, to prevent hazardous, flammable vapors from forming, The staining container was removed from the microwave oven and gently shaken the gel for 5 minutes at room temperature on an orbital shaker. The staining solution was drained and the gel rinsed with deionized water and the stained gel was placed in a staining container containing 100 ml destaining solution while, covered and heated in a microwave oven at full power for 8 minutes after which the destaining solution was drained and fresh destaining solution was added. The gel was gently shaken at room temperature on an orbital shaker until the desired background is achieved.

Statistical Analysis

All experiments were conducted in triplicates, and results were expressed as mean ± standard deviation. Statistical analysis was carried out using two-ways Analysis of Variance (ANOVA) in SPSS software, and significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

Molecular weight of lipase produced

Plate I revealed the molecular weights of lipase produced by *B. subterraneous*CW27 - B11 (B1) and crude protein (C1, C2) using SDS- PAGE. The result shows that purified lipases of *B. subterraneous*CW27 - B11 had molecular weight of 25kDa while that of the crude enzymes had different weights ranging from 26 – 100kDa.

In this study, the SDS-PAGE analysis of the purified lipase enzyme from abattoir soil isolates, a prominent band was observed at approximately 25kDa, corresponding to the expected molecular weight of bacterial lipases. Crude extracts showed multiple bands, indicating the presence of other

cellular proteins. Following purification, a single intense band remained, confirming the successful isolation of the target protein. The intensity and clarity of the band suggest high expression levels and reasonable purity. The comparison with the protein marker verified the molecular size, consistent with known lipases from *Bacillus* spp (Zarinviarsagh *et al*., 2017). The SDS-PAGE profile confirmed the successful isolation and purification of lipase, as evidenced by the appearance of a single major band in the purified sample. The estimated molecular weight of 25kDa aligns with previously reported bacterial lipases from *Bacillus* species. The presence of multiple bands in the crude extract highlights the complexity of the initial protein mixture before purification (Gricajeva *et al.*, 2016)

These findings confirm the identity and relative purity of the enzyme and suggest that the purification process employed (e.g., ammonium sulfate precipitation, dialysis, chromatography) was effective.

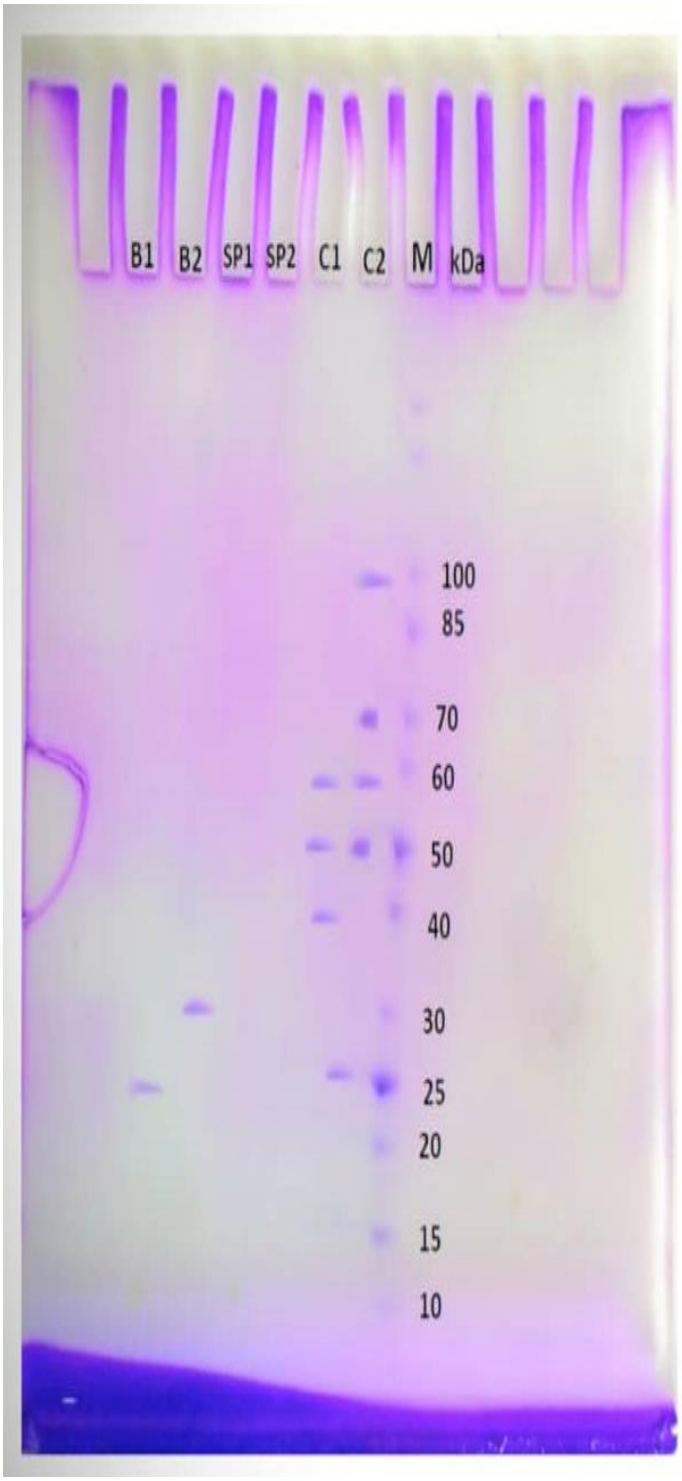

Plate 1: The SDS-PAGE Analysis of Lipase Enzyme

Key: B1= Purified lipase of *B. subterraneous*, B2= Purified lipase of *P. alcalifaciens* P3, C1= Crude enzyme and C2= Crude enzyme

The production of lipase was optimized by varying several fermentation conditions and environmental parameters, including carbon sources, inducers, nitrogen sources, pH, inoculum level, incubation period, moisture content, and temperature (Table 1).

The optimization of fermentation medium with inducer substrates enhances production and promotes its utilization since the carbon chain moity of the fatty acids present in triacylglycerol controls lipase production (Sethi *et al*., 2016). In terms of carbon source, fructose with groundnut husk stimulated the highest production of lipase (5.16±0.10U/mL) as compared to other substrates and carbon sources tested (lactose, maltose, and Fructose), followed by lactose (5.05±0.10U/mL). Glucose showed lower enzyme activity. The high yield may be attributed to its triglyceride composition, which enhances lipase gene expression in lipolytic organisms

Among the inducers (olive oil, coconut oil, groundnut oil), groundnut oil showed significant enhancement in lipase activity, with all the waste substrates (wheat bran, rice husk and groundnut husk) with groundnut husk and groundnut oil having stimulated higher lipase (26.00U/mL) Groundnut oil can increase cell permeability and enhance substrate availability, thereby promoting lipase secretion, the high amount lipase production observed in the presence of groundnut oil may be due to high oleic and linoleic acids in the edible oil that act to enhance lipase activity (Santos *et al*., 2014). The decrease in lipase production observed in coconut oil as well as olive oil may probably be due to variations in the fatty acid composition of the oils which play a key role in the enzyme production (Krzyczkowska and Kozlowska, 2017). Maximum lipase production with coconut oil by *P.roquefurti* by (Venkatesagowda *et al.,* 2015), *Micrococcus rosue* (Joseph *et al.,* 2011) in the presence of groundnut oil and inducible substrate has been reported. Rohit *et al.* (2011) also reported that the lipase production was more when groundnut oil, coconut oil, cardamom and gingili oil, were used as inducers. This indicates that aside olive oil used as inducers by most researchers, other oils have proven to be promising in inducing lipase production.

The nitrogen sources varied shows that urea produced more lipase with all the wastes substrates followed by ammonium nitrate, wheat bran (7.25±0.14 U/mL), rice husk (7.63±0.15 U/mL) groundnut husk (7.80±0.15 U/mL), groundnut husk with groundnut oil extract resulted in higher lipase activity compared to inorganic sources like ammonium nitrate. Organic nitrogen sources provide essential amino acids, vitamins, and growth factors, which support microbial growth and enhance enzyme synthesis. Ilesanmi *et al*. (2020) optimized the production of lipase using various nitrogen sources and concluded that Ammonium nitrate and yeast extract influences the production of higher lipase. The optimum pH for lipase production was found to be pH 6.0(6.90±0.10 U/mL). The high lipase activity was exhibited in the pH of 5-7 while optimum activity was recorded at pH 5 for both wheat bean and rice husk while groundnut husk recorded pH 6 as the optimum, in the acidic medium, reduction of lipase production was observed. The present report shows that the organism is an alkalophilic bacterium. Bacteria with lipase producing ability have been reported to prefer pH around 7 by Gupta *et al*. (2004).A neutral pH favors enzyme production by most bacteria and fungi. Deviations from the optimal pH affect the enzyme structure and microbial metabolism, leading to reduced production.

The highest lipase production was achieved at an inoculum level of 4 (6.56±0.13U/mL). Higher inoculum levels led to a slight decrease in activity. The optimum lipase produced for groundnut husk was at 4-5ml, the inoculum volume is inversely proportional to lipase activity because cells are struggling for nutrients, subsequently, the nutrients becomes exhausted and subsequent reduction of lipase production probably because of nutrients depletion and waste accumulation. Salihu *et al*. (2012) reported that inoculum volume has direct proportional effect on lipase activity. The findings in this work supported this claim as the medium inoculated with the 3ml culture volume, showed highest lipase activity. However, Thakur *et al.* (2014) reported inoculum volume is inversely proportional to lipase activity because cells are struggling for nutrients. Reduced activity was observed with lower and higher inoculum levels which may be attributed to insufficient biomass leading to reduced product formation and too much biomass leading to the poor product formation (Sarat *et al*., 2010). Adequate inoculum size ensures sufficient biomass for active metabolism and enzyme production. Excessive inoculum may lead to nutrient depletion and accumulation of toxic metabolites, which could inhibit enzyme synthesis.

Maximum lipase activity was recorded after 48hours (7.22±0.14) of incubation in groundnut oil substrate and 30% (7.26±0.15) in rice husk substrate, with a decline observed after prolonged incubation. The peak production at 48 hours suggests that lipase is a secondary metabolite, synthesized during the stationary phase. Prolonged incubation may lead to lipoytic degradation of the enzyme or nutrient exhaustion. The decline could be related to consumption of nutritional elements. Gupta *et al*. (2004) reported that maximum lipase activity occurs from few hours to several days.

An optimal moisture content of 40% (8.25±0.17) supported maximum enzyme production. Both higher and lower moisture levels resulted in decreased activity.Adequate moisture is critical in solid-state fermentation to ensure nutrient solubility and microbial mobility. Excessive moisture can reduce porosity and oxygen diffusion, while low moisture limits microbial growth and metabolic activity.

The optimum temperature for lipase production was found to be 40%, with significant activity observed between 40°C40 (7.08±0.14) and 50ºC. The optimal temperature corresponds to the mesophilic nature of the producing organisms. At higher temperatures, enzyme denaturation or cell viability issues may occur, while lower temperatures may slow down microbial metabolism and enzyme synthesis, the decrease may probably be because of nutrients depletion and waste accumulation. In SSF system temperature is also of much importance because of its effect on microbial growth and enzymes production. During fermentation, the temperature of the fermenting mass is generally increased due to respiration. At higher temperature low enzymes production was seen because of substrate drying. Several researchers have reported optimum temperature for lipase production to be 30ºC to 40ºC

Table 1: Summary of Optimal Mean of Lipase (U/mL) Production by *B. subterraneous*CW27 - B11

Parameters	Wheat Bran (U/ml)	Rice Husk (U/ml)	Groundnut husk (U/ml)
Carbon sources	Lactose (5.05±0.10)	Maltose (4.77±0.10)	Fructose (5.16±0.10)
Inducers	Groundnut oil (8.00±1.00)	Groundnut oil (17.00±1.00)	Groundnut oil (26.00±1.00)
Nitrogen sources	Urea (7.25±0.14)	Urea (7.63±0.15)	Urea (7.80±0.15)
pH	pH 5 (6.14±0.12)	pH 5 (3.73±0.07)	pH 6 (6.90±0.10)
Inoculum level (ml)	3 (5.45±0.11)	1 (3.73±0.07)	4 (6.56±0.13)
Incubation period (hr)	24 (6.20±0.13)	48 (6.62±0.13)	48 (7.22±0.14)
Moisture content (%)	40 (7.04±0.14)	30 (7.26±0.15)	40 (8.25±0.17)
Temperature (ºC)	50 (6.38±0.13)	40 (7.08±0.14)	50 (6.98±0.14)

CONCLUSION

The SDS PAGE analysis effectively confirmed the successful purification and characterization of the lipase enzyme produced by the selected isolate from abattoir soil. The distinct protein band observed at approximately 25kDa for *B. subterraneous* CW27 - B11 in the purified sample corresponds to the expected molecular weight of microbial lipase, indicating high purity and successful analysis.. This study successfully optimized lipase production by *Bacillus subterraneus* using agro-allied wastes in SSF. Different variables such as carbon sources, inducers, nitrogen sources, pH, temperature, etc which facilitated increased lipase production by *B. subterraneous* CW27 - B11 was achieved via optimization of growth and physiological parameters with groundnut husks substrate. Highest lipase (26.00 ±1.00) was produced by *B. subterraneous* CW27 - B11 using groundnut husk as substrate. The results emphasize the potential of using agro wastes and abattoir soil isolates in developing sustainable biotechnological applications.

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