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RESPONSE SURFACE OPTIMIZATION OF LIPASE PRODUCTION BY *PSEUDOMONAS* SP. ON A LOW-COST SHEA-NUT CAKE USING SOLID STATE FERMENTATION

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ABSTRACT

Shea nut cake (SNC) is the solid residue produced after the shea butter extraction process. The SNC could provide a low-cost substrate for bacterial lipase production. Greater yield are among the benefits Solid State Fermentation (SSF). However, there have been less instances of lipase production by bacteria than fungi in SSF. The current study aimed at improving bacterial lipase production on SNC in SSF using Response Surface Methodology. Lipase-producing bacteria (LPB) were isolated from the shea butter mill effluent pond and screened for lipase production on a tributyrin agar plate. The influence of four independent parameters (temperature, pH, moisture content and biosurfactant) on lipase production was studied using Box Behnken Design (BBD) of RSM. Results showed that isolate AO (*Pseudomonas sp.)* displayed the highest zone of hydrolysis (53 mm). A quadratic regression with R^2= 0.931 showed that the model is best fitting, and predicted the optimum conditions of significant (P>0.05) growth parameters, including temperature at 40°C, moisture content at 50%, and biosurfactant at 2 mL, which produced 408.25 U/g of experimental lipase. The findings indicate that the model was useful for predicting optimal growth conditions for incresing lipase production in SSF and the low-cost agro-industrial SNC could be a promising substrate for industrial applications.

Keywords: Lipolytic bacteria; Shea butter mill waste; Solid state fermentation; Response Surface Methodology, Box Behnken Design.

INTRODUCTION

Enzymes are biologically efficient catalysts that facilitate the degradative reactions of living organisms. Lipases are a family of lipid degrading enzymes that has garnered a lot of interest in research and industries. They are triacylglycerol acetylhydrolases belonging to the serine hydrolase family and known as carboxylic acid esterase (EC 3.1.1.3), capable of hydrolyzing fats and oils (without co-factor) into diglycerides, monoglycerides, fatty acids and glycerol (Nomwesigwa *et al*., 2023). It is one of the most well-known biocatalysts, making up to 10% of the enzyme market (Nema *et al*., 2019). Their efficiency in catalyzing a variety of reactions such as esterification and transesterification, as well as their capacity to function in both aqueous and non-aqueous solvent systems, makes them highly desirable industrial biocatalysts (Nomwesigwa *et al*., 2023). Microbial lipases are more valuable than plant-based lipase because of their various catalytic activity, high yield, simplicity of genetic manipulation, consistent supply, stability, safety, and fast growth rate. (Chandra *et al*., 2020). Bacteria, fungi, and yeast produce lipase through fermentation, with bacterial sources being more suitable due to their resistance to harsh industrial conditions, versatility, and higher yield (Javed *et al*., 2018). Recent reports indicate the production of lipase by bacteria, including *Lactobacillus fermentum* (Fathi *et al*., 2022), *Bacillus safensis* (Patel and Parikh, 2022), *Bacillus salmalaya* (Al Mohaini *et al*., 2022), *Pseudomonas plecoglossicida* (Choudhary *et al*., 2023) and *Bacillus velezensis* (Kazeem *et al*., 2024). Lipase has found usefulness in various industries, including biodiesel, waste cooking oil treatment, detergent, food and bioremediation (Phukon *et al*., 2020; Zhao *et al*., 2022; Sutar *et al*., 2023).

Microbial enzymes can be produced through Solid States Fermentation (SSF) and Submerged Fermentation (SmF). The SSF involves growing microorganisms on solid substrates without free water, forming a fine particle layer (Araujo *et al*., 2022). The organism consumes micronutrients and excretes metabolites. However, SmF uses free-flowing liquid substrates for higher moisture-content microorganisms (Akinduyite *et al*., 2022). Solid-state fermentation, due to the absence of water, requires less microbial sterility, enhances fermentation productivity, and increases product

concentration (Szymczak *et al*., 2021). Agro-industrial wastes such as oil cakes from sunflower, cottonseed, sesame, mustard, groundnut, palm kernel, and olive trees were used in the synthesis of lipase due to their high fat, protein and carbohydrate content (Sarkar *et al*., 2021). However, little attention have been paid to the use of SNC as a potential substrate for lipase production. The SNC is an oily solid waste generated during the production of shea butter extracted from the almonds of *Vitellaria paradoxa*. SNC accumulation in mills is an environmental burden. As a result, SNC could serve as a potentail substrate for lipase production. Recently, the addition of SNC to olive oil has been shown to enhance lipase production by *B. velezensis* (Kazeem *et al.,* 2024). Improvement of lipase production is vital in industrial bioprocessing and this can be achieved through optimizing the growth parameters affecting lipase production. The RSM is a mathematical and statistical prediction tool that evaluates the influence of independent variables on responses with fewer tests (Hammoudi *et al*., 2019), unlike one-factor-at-a-time (OFAT) methods, which have limitations such as the inability to predict interactions and large experimental runs, making them unsuitable for large-scale research (Elhussiny *et al*., 2023). The RSM has been used previously as a tool to enhance the production of enzymes such laccase (Arekemase *et al.,* 2023) and xylanase (Kazeem *et al*., 2023).

Therefore, the current study aims to investigate the potential of *Pseudomonas* sp. grown on SNC in a SSF and improve the lipase production through response surface optimization.

MATERIALS AND METHODS

Sample collection

A sludge sample was collected from a shea butter mill effluent pond at a shea butter factory in Apa Ola's local community in Kwara State, Nigeria.

Isolation of lipase-producing bacteria (LPB)

A 5% (v/v) effluent sample was transferred into a 250 mL Erlenmeyer flask containing 100 mL of enrichment medium (EM) with olive oil as the sole carbon source and incubated at 40°C for 5 days at 150 rpm. The composition of the enrichment medium included olive oil 1% (v/v), NaCl 0.1% (w/v), $MgSO_4.7H_2O$ 0.02% (w/v), $MgCl_2.6H_2O$ 0.035% (w/v), $CaCl_2.2H_2O$ 0.025% (w/v), KH_2PO_4 0.015% (v/v), K_2HPO_4 0.015% (v/v), $(NH_4)_2SO_4$ 0.025% (w/v). After five days, 1 mL aliquot of serially diluted sample from the enrichment medium was inoculated onto an olive oil agar (OOA) plate containing 13 g/L nutrient broth, 15 g/L agar, and 10 mL olive oil and incubated at 40°C for 24-48 h. Pure cultures were obtained and stored at 4°C (Kazeem *et al.,* 2024).

Screening and selection of LPB

The pure isolates were screened for lipase activity using Tributyrin agar [5 g/L peptone, 3 g/L yeast extract, 15 g/L agar, 10 mL tributyrin oil, 2.5 g/L of $CaCl_2$, and 5 g/L $MgCl_2$] plates as described by Carrazco-Palafox *et al.* (2018). Thereafter, a 2 mm cork borer was used to create a well at the center of the plates. Aliquots of 0.1 mL from a 24 h-old bacterial suspension in nutrient broth were inoculated into the wells under aseptic conditions and incubated at 40°C for 3 days. Visible clear zone resulting from hydrolysis of tributyrin shows the presence of lipolytic activity. Lipase-positive isolates were selected for further analysis.

Temperature tolerance and identification of LPB

The LPB were cultured on the OOA plate and incubated at temperatures ranging from 35-60°C for 24 h. After 24 h, the level of growth was observed visually and the most tolerant isolate was identified through biochemical characterization.

Lipase Assay

Lipase activity was measured using colorimetry method described by Kwon and Rhee (1986). In a water bath shaker, culture filtrate (1 mL) was agitated with 2.5 mL of olive oil emulsion (1:1, w/v) and 0.02 mL of 0.02 M $CaCl_2$ at 200 rpm for 30 min at 50°C. To make the emulsion, an equal volume of olive oil and 0.05 M phosphate buffer (pH 7.0) were combined and stirred with a magnetic stirrer for 10 min. The reaction was halted by adding 1 mL of 6 M HCl and 5 mL of isooctane, followed by stiring with a vortex mixer for 30 s. A 4 mL sample was withdrawn from the upper layer and mixed with 1 mL copper reagent for 30 s. The absorbance of the upper layer was read at 715 nm. Lipase activity was determined by

measuring the amount of free fatty acids released based on the standard curve of free fatty acids. One unit of lipase activity was defined as the amount of enzyme releasing 1 mole of fatty acid per min under the specific assay condition.

Optimization of lipase using Response Surface Methodology (RSM)

The physical parameters were altered using the BBD under the RSM to study the influence of growth parameters on lipase production. The RSM used a mathematical model to assess the relationship between the parameters to determine their importance in lipase production. A total of 4 variables comprising of growth factors (Temperature (°C), pH, moisture content (%), and bio-surfactant (mL)) at 3 levels were studied and the values of each factor is as shown in (Table 1).

Table 1: Experimental design of independent variables used in the BBD

Variables/ Range	Low	Medium	High
Temperature (°C)	35	50	65
pH	6	8	10
Moisture content (%)	30	50	70
Biosurfactant (mL)	0.05	1.25	2.0

Enzyme Extraction

After incubation, 40 mL of phosphate buffer (pH 7.0) was added to the solid culture, which was then agitated at 180 rpm for 60 min at 50°C and filtered using Whatman No.1 filter paper. The filtrate was centrifuged for 15 min at 10,000 rpm, and the supernatant was employed in the lipase experiment.

Statistical Analysis

Analysis of Variance (ANOVA) was performed on the data obtained using MINITAB® 17 software (Mini-Tab LLC, Pennsylvania, USA). Values of $p<0.05$ were considered significant.

RESULTS

The purpose of this study was to investigate the optimization of lipase production by potential LPB isolated from shea mill effluent using shea nut cake (SNC) as substrate. The OA isolate produced the highest lipase clear zone (5.3 mm) on the tributyrin agar plate and was selected for the optimization studies. The biochemical characterization (Table 2), showed that he bacteria is a gram negative rod with greenish pigmentation and suggested to be *Pseudomonas* sp. Molecular identification will further confirm its related specie.

Table 2: Morphological and biochemical characterization of isolate OA

Test	**Result**
Morphological features	
Colony	Irregular, curled, flat, opaque, greenish and small
Gram's staining	-ve, rods
Biochemical	
Citrate utilization	+
Catalase	+
Sugar Utilization	
Glucose	+
Sucrose	+
Lactose	+
Fructose	+
Mannitol	+
Starch	+

To investigate tolerance of the bacteria to temperatures, the bacterial isolate was exposed to multiple temperatures (Table 3). Results suggested that the *Pseudomonas* sp. was more tolerant to temperatures between 35-40°C. Temperatures above 40 °C negatively affected the growth rate of the bacteria.

Table 3: Temperature tolerance test for isolate OA

Temperature(⁰C)	Growth rate
60	+
50	+
40	+++
35	+++

Key: + =low growth rate, +++ = high growth rate

The BBD was used to determine the optimum concentration and interaction among 4 factors (Temperature, pH, moisture content and bio-surfactant) that could possibly influence lipase production by the *Pseudomonas* sp. A total of 54 runs were generated as presented in Table 4. The highest lipase production (430.5 U/mL) was obtained from run 51 while the lowest lipase prodcution (50.5U/mL) appeared in run 46.

Table 4: Box- Behnken experimental design matrix for optimization of lipase production using Shea nut cake as substrate by *Pseudomonas sp.*

Run Order	Temperature (°C)	Moisture (%)	pH	Biosurfactant (mL)	Lipase activity (U/g)
1	50	50	6	0.050	275.5±0.003
2	50	50	8	1.025	289.5±0.012
3	35	50	8	0.050	333.5±0.011
4	65	50	8	0.050	217.5±0.023
5	65	30	8	1.025	142.5±0.017
6	35	70	8	1.025	420.0±0.019
7	35	50	6	1.025	180.5±0.051
8	65	70	8	1.025	87.5±0.042
9	50	70	8	2.000	194.0±0.032
10	50	50	8	1.025	286.5±0.023
11	35	50	8	2.000	217.5±0.044
12	50	50	10	2.000	301.0±0.051
13	50	70	6	1.025	224.0±0.033
14	50	30	6	1.025	104.0±0.033
15	50	70	10	1.025	217.5±0.038
16	50	70	8	0.050	199.0±0.040
17	50	50	6	2.000	106.5±0.072
18	50	30	8	0.050	192.0±0.027
19	50	50	8	1.025	158.0±0.034
20	50	30	8	2.000	125.5±0.071
21	50	30	10	1.025	293.0±0.021
22	65	50	6	1.025	51.0±0.004
23	35	30	8	1.025	78.5±0.055
24	50	50	10	0.050	286.0±0.016
25	65	50	10	1.025	195.0±0.031
26	65	50	8	2.000	51.5±0.053
27	35	50	10	1.025	422.5±0.002
28	50	50	6	0.050	267.0±0.032
29	35	50	8	2.000	214.5±0.043
30	50	30	8	0.050	389.5±0.053
31	50	70	8	2.000	223.0±0.004
32	50	50	6	2.000	80.5±0.009
33	50	70	10	1.025	220.5±0.010
34	50	70	6	1.025	222.0±0.051
35	35	50	8	0.050	409.5±0.031
36	65	50	8	0.050	169.0±0.090
37	50	50	10	2.000	142.0±0.025
38	65	30	8	1.025	154.0±0.013
39	35	50	6	1.025	200.5±0.015
40	35	30	8	1.025	207.5±0.016
41	50	50	8	1.025	410.5±0.023
42	35	70	8	1.025	401.0±0.054
43	50	70	8	0.050	301.0±0.032
44	65	50	10	1.025	173.5±0.021
45	50	50	8	1.025	158.0±0.043
46	50	30	6	1.025	50.5±0.022
47	35	50	10	1.025	427.5±0.035
48	50	30	10	1.025	223.5±0.004
49	65	70	8	1.025	113.0±0.010
50	50	30	8	2.000	85.5±0.018
51	40	50	8	2.000	430.5±0.024
52	50	50	10	0.050	278.5±0.041
53	50	50	8	1.025	410.5±0.051
54	65	50	6	1.025	96.0±0.008

A quadratic equation was derived to predict the impact of each independent variable on the response lipase in equation 1.

$Y= -2184 + 21.8A + 36.27B + 305C – 429D - 0.085A^2 - 0.1414B^2 - 11.32C^2 - 14.5D^2 - 0.2629AB - 1.077AC + 4.01AD - 1.156BC + 1.84BD + 15.0CD$ ……………..(1)

where Y is the lipase activity (U/mL) produced as a function of the coded levels of temperature (A), moisture content (B), pH (C) and biosurfactant (D), respectively.

The significance of each factor on lipase production was further investigated using ANOVA. The p-value shows the level of significance of each term while the determination coefficient (R^2) indicated a perfect coherence between the predicted and experimental values. Higher R^2 and smaller p-value suggests significant corresponding coefficient. The result from ANOVA (Table 5) showed that temperature, pH and biosurfactant had significant effects at ($P<0.05$) on lipase production with P-values of 0.000, 0.002 and 0.010, respectively. The interaction between moisture content*moisture content (B^2) temperature*moisture content (AB), and temperature*biosurfactant (AD) displayed significance with P values of 0.029, 0.009 and 0.048, respectively which was less < 0.05. The model established that the computed F-value for the experiment was 4.34 with a P-value of < 0.0001, implying the model is significant. Furthermore, the R^2 value was used to measure the robustness of the model as it shows how good a prediction and fit of the model. In the current study the model showed an R^2 value of 0.9313. The "predicted R^2" (0.9313) was in a reasonable agreement with the "adjusted R^2" (0.9857). Similarly, the adjusted R^2 value showed a variation of 98.57% in lipase activity, attributable to the independent values, and only 1.43% of the total variation could not be explained by the model. The resulting model makes it easier to predict the individual and interactive effect of physical growth parameters on the organism's production of lipase.

Table 5: Analysis of variance for the quadratic response surface model

Source	DF	Adj SS	Adj MS	F-Value	*p*-value
Model	15	430299	28687	4.34	0.000*
Blocks	1	13728	13728	2.08	0.158
Linear	4	252214	63053	9.53	0.000*
Temperature (A)	1	104083	104083	15.74	0.000*
Moisture (B)	1	25123	25123	3.80	0.059
pH (C)	1	74093	74093	11.20	0.002*
Biosurfactant (D)	1	48916	48916	7.40	0.010*
Square	4	44450	11112	1.68	0.175
A^2	1	3906	3906	0.59	0.447
B^2	1	34139	34139	5.16	0.029*
C^2	1	21851	21851	3.30	0.077
D^2	1	2038	2038	0.31	0.582
2-Way Interaction	6	119907	19985	3.02	0.016
AB	1	49770	49770	7.52	0.009*
AC	1	8353	8353	1.26	0.268
AD	1	27495	27495	4.16	0.048*
BC	1	17113	17113	2.59	0.116
BD	1	10332	10332	1.56	0.219
CD	1	6845	6845	1.03	0.315
Error	38	251332	6614		
Lack -of-Fit	34	197557	5810	0.43	0.923
Pure Error	4	53775	13444		
Total	53	681631			

$R^2 = 0.9313$ R^2 (adj) =0. 9857 *= significant

To further investigate the interaction effect of each factor on the response, 3D surface plots were plotted. The effect of temperature and moisture content and their interactions on lipase production (Figure 1) showed that both factors contribute significantly towards lipase production. The optimum temperature for lipase production was attained at 40°C with a moisture content optimal at 50%. However, maintaining the temperature at 40°C while increasing the temperature beyond 50°C will lead to a decrease in lipase production. The interactions between temperature and biosurfactant (Figure 2) revealed that optimal lipase was reached at reached 40°C and a peak of 2 mL biosurfactant concentration. Decrease in the concentration of biosurfactant with concomitant increase in temperature from 40 to 60 °C resulted in a decline in lipase production, which further reveal the significance of the interaction between incubation temperature and biosurfactant concentration. The RSM's optimized condition was validated through an experiment in a static flask, revealing an increase in lipase production to 408.25 U/g, close to the predicted 423.15 U/g.

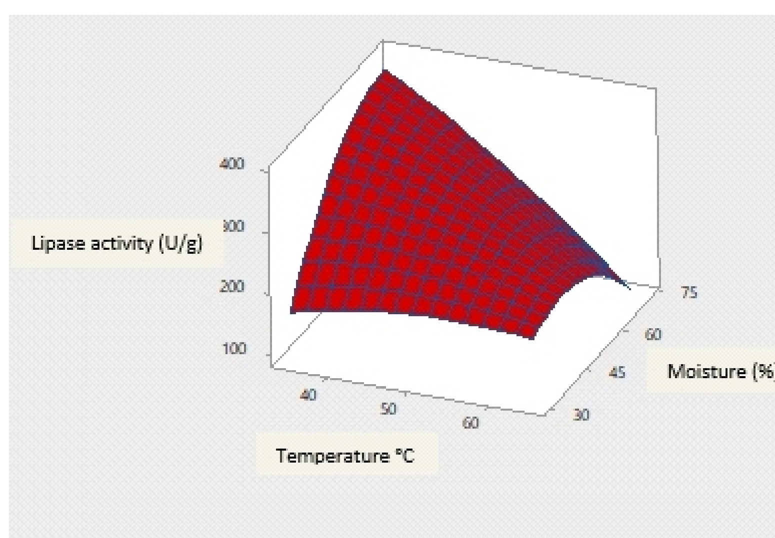

Figure 1: 3D surface plot showing the interaction effect of temperature and moisture content on lipase production

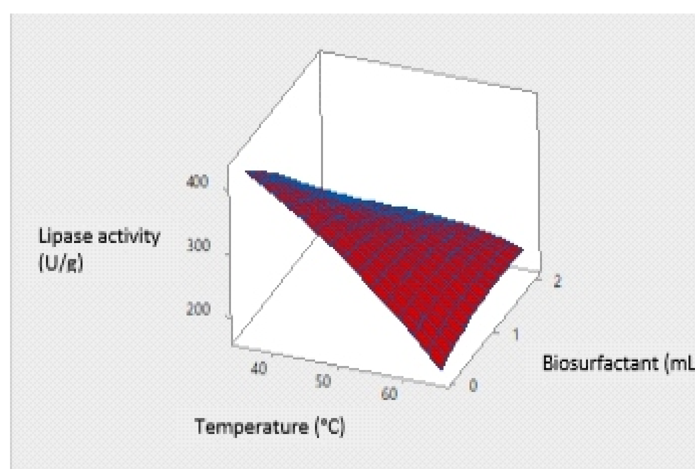

Figure 2: 3-D surface plot showing the interaction effect of temperature and biosurfactant on lipase production

DISCUSSION

Lipase-producing bacteria have been isolated from a variety of habitats, including oil-contaminated soil, industrial waste, dairy facilities, oil processing plants, and compost heaps. . Commercially valuable ones are related to the genera *Burkholderia, Bacillus, Arthrobacter, Achromobacter, Alacligenes, and Pseudomonas.* (Pham *et al.*, 2021). The study examined the lipase-producing capacity of bacteria from shea mill effluent, cultured using SNC as a substrate. Danikuu and Sowley (2014), previously reported SNC degradation by indigenous soil bacteria in a culture medium supplemented with SNC. In this study, the bacteria strain OA was isolated from the shea mill effluent using an enrichment medium supplemented with 1% olive oil. This enrichment process is required to promote the growth of LPB. The observed zone of hydrolysis (5.3 mm) was higher than that reported for *B. velezensis* (1.9mm) isolated from hot compost (Kazeem *et al.*, 2024). Biochemical characterization of isolate OA revealed that isolate OA belongs to the genus *Pseudomonas sp*. Similar to this study, , Ibiyemi and Julius (2022) discovered that *Pseudomonas aeruginosa*, *P. syringae,* and *P. putida* from palm oil processing sites have a higher lipase production capacity. Similarly, *Bacillus amyloliquefaciens*, which produces lipase, has been identified from soil samples from oil refineries, auto garages, and residential waste areas (Mazhar *et al.*, 2023). To the best of our knowledge, this is the first report on LPB in shea mill effluent.

Temperature regulates microorganism development through metabolic activities, influences cell structure, and external enzyme excretion. Cultivating microorganisms at optimal development temperatures leads to increased productivity. In the current study, temperature tolerance at 35-40°C demonstrated by *Pseudomonas* sp. reveals the optimal state, while diminished growth at higher temperatures reflects the degree of tolerance. Temperatures that exceed the bacteria's tolerance cause structural and physiological damage to the cell. As a result, incubation temperatures of 50 and 60 °C may promote delayed growth and death of the bacteria. SNC was employed as a carbon source for lipase production, making it a less expensive option. The optimum lipase activity attained at 40°C agrees with Rasmey *et al.* (2017), who found that lipase production by *Pseudomonas monteilli* had an optimal temperature of 40°C However, Sattar and Ali (2019) reported maximum lipase production by *Pseudomonas aeruginosa* at 30°C. Higher temperature accelerates enzymatic reactions in cells, while protein denaturation slows metabolism, affecting cell proliferation and productivity (Ali *et al.*, 2021). Because water is present in limited amounts in SSF, it is vital to identify the required moisture level for optimum productivity in the SSF. Moisture content higher or lower than the organism's requirement impacts metabolic activity and enzyme production. Similar to this study, Kudari (2017) study found that *Pseudomonas aeruginosa*, cultivated on agro-industrial waste, requires 50% moisture content for lipase production.. Moisture levels below or above 50% may cause asphyxia and contamination. Like moisture content, pH is a crucial factor influencing lipase production in SSF. *Pseudomonas* sp. in this investigation produced lipase at an ideal pH of 8.0. There have been reports of various pH ranges optimal for lipase production. For instance pH of 6.0 was reported optimum for lipase production by *P. aeruginosa* PseA grown on jatropha seed cake (Mahanta *et al.*, 2008). In the case of a *Pseudomonas* sp., an optimal pH of 5.0, 6.0, and 9.0 was reported on different agro-waste substrates (Jamilu *et al.*, 2022).

From the ANOVA study, the R^2 value was used to measure the robustness of the model as it shows how good a prediction of the model gives to the response. The high R^2 value of the model and the closeness of the actual value to the predicted explains the suitability of the model as it could explain 93.13% variability in response. A previous study by Amin *et al.* (2018) reported a 98.91% difference in response to lipase activity produced by *Penicillium fellutanum.* Aachapa *et al.* (2021), reported a 97.11% R^2 value of distinction in lipase activity produced by *Bacillus sp.* A previous report indicated that the coefficient of determination (R^2) obtained by ANOVA is used to determine the efficiency of the model and that if the value is closer to 1, the model is fit since there is a strong correlation between the predicted and observed values (Geoffry and Achur, 2018). Among all the factors considered, temperature showed the greatest significant effect at ($p < 0.05$) on lipase

production, followed by pH, while the lipase production was least affected by the biosufactant. This implies that all these factors significantly impact on the lipase production by the *Pseudomonas* sp. The level of parameter significance recorded in this study agrees with the report of Nadeem *et al.* (2021). The pH changes can affect the permeability of cell membrane as well as the stability of the enzyme. The pH values above or below the optimal can suppress ionization in the fermentation medium, reducing nutrient uptake and product formation (Kazeem *et al.*, 2023).

The 3D response surface plot enables the analysis of the interactions between significant variables and their impact on lipase production (Kazeem et al., 2024). Significant variables at (p>0.05), e.g., temperature, moisture and biosurfactant and their interaction were studied to ascertain maximum lipase production from *Pseudomonas* sp. According to the investigation, under the optimal condition of 2 mL biosurfactant, temperature at 40 °C and 50 % moisture content, maximum lipase activity of 408.25 U/g was produced on SNC. The experimental design and analysis effectively identified crucial parameters for optimal conditions for maximum lipase production. Improvement in lipase production by *Pseudomonas* sp. using the BBD-RSM, was considerably higher than some reported in previous similar investigations. For instance, according Sahoo *et al.* (2018) optimal lipase production at 62.3 U/g produced from *B. licheniformis* was obtained at temperature 50 °C, 50% moisture content, and 1.025 g biosurfactant on an olive oil cake. In another investigation, maximal lipase production of 180.75 U/g at 28 °C, pH 5.9, and 33% moisture content from a ground nut cake was reported utilizing the BBD (Faisal *et al.*, 2014). Similarly, a recent work by Kazeem et al. (2024) on optimizing growth factors on lipase production by *Bacillus velezensis* EAC9 cultured on SNC resulted in the enhancement of lipase activity up to 200 U/mL, which was lower than that reported in the current study. The difference in lipase yield could be attributable to a variety of factors such as the organism's nature, substrate type, the components optimized, and the optimization model used.

CONCLUSION

In this study, *Pseudomonas* sp. isolated from a shea mill effluent pond was efficient in lipase production. Improvement in lipase production to 408.25 U/g was achieved at optimal conditions employing the BBD-RSM. The optimization studies revealed that interaction between temperature, moisture content and biosurfactant significantly affected lipase production by *Pseudomonas* sp. on SNC. The model used was useful and fit the prediction of suitable parameters for lipase production. The study further shows the potential of SNC as a low-cost substrate supporting lipase production in low-energy-demanding solid-state fermentation. As a result, a baseline for industrial lipase production by *Pseudomonas* sp. is established.

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CONFLICT OF INTEREST

The authors declared they have no conflict of interest

AUTHORS' CONTRIBUTIONS

K.M.O. designed the work; M.M.A. carried out the research; K.M.O. and M.M. A. analyzed and interpreted the results; M.M. A., K.M.O. and M.D. wrote the manuscript; K.M.O. and M.D. proofread the manuscript.

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