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OPTIMIZATION OF LIPASE FROM BACTERIA ISOLATED FROM SOIL

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ABSTRACT

Lipases catalyze the hydrolysis of triacylglycerols to glycerol and fatty acids. Microbial lipases are receiving much attention because of their industrial potential. This study focuses on optimization of lipase from bacteria isolated from soil. Using pour plate method for isolation, the lipase producing isolate was identified and optimization of the effect of different parameters (pH and temperature) on the activity of lipase produced was evaluated. *Bacillus subtilis* was identified as the lipase producer. The optimization of pH and temperature showed slight effect on the activity of lipase. Lipase activity was observed to be maximal at optimum pH 8.0 as 1.681µmol/min and maximal at optimum temperature 50°C as 1.504µmol/min. As pH and temperature increases respectively lipase activity reduces p-value < 0.05. *Bacillus subtilis* from this study demonstrates remarkable lipase producing potential. Further study of the characterization and purification for industrial and environmental applications particularly toward bio treatment of oil-contaminated and polluted environment is essential.

Key words: Bacteria; Lipase; Optimization; pH; Soil; Temperature.

INTRODUCTION

Enzymes are considered as biological catalysts. Most enzymes today (and probably nearly all in the future)are produced by the fermentation of bio-based materials. Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these water-insoluble compounds (Hasan *et al.*, 2006). Lipolytic enzymes are involved in the

breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another. Several enzymes possessing different substrate specificities are known, however only comparatively few enzymes have been isolated in a pure form and crystallized, and little has been known about their structure and function. However, the advent of protein engineering techniques makes their application to important as industrial enzymes, such as proteases and lipases used in detergents, amylases and glucose isomerase used in starch processing and in the bio processing of raw materials or in the synthesis of organic chemicals (Cheetham, 1995).

Lipase is an ester hydrolase, which catalyse the hydrolysis of triacylglycerol to glycerol and fatty acids (Sharma *et al.*, 2001). These enzymes are ubiquitous in nature and are found in animals, plants, fungi and bacteria. Substrate specificity and otherenzymatic properties, such as broad sources, short cycle, wide pH, wide range of temperature, microbial lipase had played a more important role than animal and plant lipases in enzymatic theoretical research as well as practical and industrial application, including hydrolysis, esterification, trans esterification, and ester chiral synthesis (Joseph*et al.*, 2008).The ability of lipases to perform very specific chemical transformation (biotransformation) has make them increasingly popular in the food, detergent, chemicals and pharmaceutical industries (Gupta *et al.*, 2007; Grbavic*et al.*, 2007).

Lipases are produced by many microorganisms and higher eukaryotes. Most commercially useful lipases are of microbial origin. Microbial lipases are more stable than their corresponding plant and animal origin and their production is more convenient, safer and can be obtained in bulk at low cost (Vakhlu and Kour, 2006). Generally, it well known that bacterial enzymes are more preferred over fungal enzymes because of their higher enzymatic activities in neutral or alkaline pH. In order to increase the cell yields and the enzymatic activities of the cells or to produce altered enzymes, genetic and environmental manipulations can be performed more readily on bacterial cells due to their short generation times, simple nutritional needs and easy screening procedures for desired properties (Hasan *et al.*, 2006).A variety of lipases are produced from both Gram-positive and Gram -negative bacteria, however, greater part of these enzymes comes

from Gram-negative bacteria. The most important Gram-negative bacteria genus is *Pseudomonas* which contains at least seven lipase producing species namely: *Pseudomonas aeruginosa, Pseudomonas alcaligenes, Pseudomonas fragi, Pseudomonas glumae, Pseudomonas cepacia, Pseudomonas fluorescens and Pseudomonas putida* (Jaeger *et al.*, 1994; Kojima *et al.*, 2003). Besides *Pseudomonas* species, *Achromobacter, Alcaligenes, Burkholderia* and *Chromobacterium* strains are the most common lipase producing Gram-negatives (Gupta *et al.*, 2004).

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseed and decaying food, compost heaps, coal tips and hot springs (Dutra *et al.*, 2008). Lipase producing microorganisms include bacteria, fungi, yeasts and actinomyces etc, and originated mostly from plants, animals, fungi, yeast and bacteria (Kumar and Gupta, 2012). They all show highly specific activity towards glyceridic substrates (Hasan *et al.*, 2006). This present study was focused on the optimization of lipase from bacteria isolated from soil, due the ever increasing demand for better lipases in the industrial sector and the search for novel sources of lipase (Vijay and Das, 2005).

MATERIALS AND METHODS

Sample Collection

The soil samples were collected behind the Microbiology Laboratory using a sterile spatula from the depth of 20 to 25cm surface sampling system as reported by the National Association of testing Authorities (NATA, 2005).Samples were then immediately transferred into the Microbiology Laboratory, Federal University of Technology, Minna, Nigeria and stored at 4°C in the refrigerator until analysis was conducted.

Experimental setup

The experiment was carried out in the Microbiology Laboratory, Federal University of Technology, Minna, Nigeria. After the soil sample collection, serial dilution, inoculation of

nutrient agar using pour plate method, screening of isolates for lipase production using tributyrin agar, Gram staining and biochemical test, production of crude enzyme using mineral salt medium with mustard oil, optimization of effect of pH and temperature on the enzyme produced.

Preparation of Tributyrin agar medium

Exactly 0.5g of peptone, 0.3g of yeast extract, 1g ammonium nitrate, 0.05g of KH_2PO_4, 0.1g glucose, 2g agar weighed into a conical flask and one to two drops of tween 80, 1mL of mustard oil were added and distilled water was added to mark as described by Verma *et al.* (2014).

Isolation of bacteria

Samples of soil were diluted serially from 10^{-1} up to 10^{-6} in sterile distilled water, dilution factor 10^{-5} were inoculated on nutrient agar plates by pour plate method to obtain colonies after 24 hours of incubation at 37°C (Kumar and Gupta, 2012). Plates with highest number of colonies were estimated for CFU/g counts after incubation.

Screening for lipase activity

Pure bacterial isolates were screened for lipase production as described by Kumar and Gupta,(2012). Bacterial colonies were streaked on agar plates containing tributyrin (1%, w/v), agar (2%, w/v) at 37°C for 48 hours. Clear zone around the colonies on tributyrin agar were observed and diameter was measured indicating lipase producing bacteria

Identification of lipase producers

Lipase producing bacterial colony was observed on the basis of shape, size, texture, margin, elevation, optical property, pigmentation and Gram reaction as described in Bergey's manual of determinative bacteriology. Biochemical tests include catalase, coagulase, urease, motility, oxidase, citrate, starch hydrolysis, casein hydrolysis and mannitol were carried out (Selva*etal.*, 2008).

Enzyme production

The medium used for the production of the crude enzyme consist of 0.2g0.1g; 0.25g, 0.04g, 0.04g of peptone, $NH_4H_2PO_4$,NaCl, $MgSO_4 \cdot 7H_2O$ and $CaCl_2.2H_2O$ respectively with 2.0 mL mustard oil, 1-2 drops Tween 80 as emulsifier at pH 7.0. Overnight cultures were suspended in 20mL of sterile nutrient broth and incubated at 37°C for 4-5 hrs and 2mL of the submerged microbial cultures were inoculated into 500 mL Erlenmeyer flasks containing 100 mL of liquid medium on a rotary shaker (150 rpm) and incubated at 30°C. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4°C and the cell free culture supernatant fluid was used as the sources of extracellular enzyme (Karadzic *et al.*, 2006).

Assay for lipase activity

Lipase activity was determined spectrophotometrically at 30°C using p-nitrophenol palmitate (pNPP) as substrate. The reaction mixture was composed of 700 µl*p*NPP solution and 300 µl of lipase solution. The *p*NPP solution was prepared by adding the solution A (0.001 g *p*NPP in 1mLisopropanal) into solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50 µl Triton X-100 and 9 mL of 50 mM Tris-HCl buffer, pH 8) with stirring until all was dissolved. Then the absorbance measured at 410 nm for the first 2 min of reaction. One unit (1U) was defined as that amount of enzyme that liberated 1µmol of *p*NPP per minute (ε: 1500L/mol cm) under the test conditions (Karadzic *et al.*, 2006).

Optimization of the Effect of pH and Temperature on Lipase Activity

The crude enzyme used for assay was the culture broth after separation of cells and particles. The enzyme was stored at 4°C until used. The optimal temperature for activity was determined at different temperatures (30–90°C), at pH 8.0 for 10 min and for determination of temperature stability, the reaction mixtures containing the enzyme in 50mM Tris–HCl buffer (pH 8.0) was incubated at different temperatures (30,40, 50, 70, and 90°C) for 3 h and immediately cooled. The enzyme activity was measured under standard enzyme test conditions. Optimal pH was determined at 35°C in buffer solutions of pH values ranging from 6 to 10 (0.1 HCL,0.1 NaOH). The effect of pH on enzyme stability was analyzed by the spectrophotometric assay after pre-

incubation of 300µL of enzyme solution for 1 h at 30°C, in 700µLof the above mentioned buffer solutions (pH 6–10). Enzymatic activity was measured according to a standard protocol with *p*NPP as the substrate (Karadzic *et al.*, 2006).

Data Analysis

The data was analysed using One-Way ANOVA ("analysis of variance") and regression analysis which shows the relationship between the dependent variable and the independent variables using the SPSS application.

RESULTS

Isolation

Colonies on Nutrient agar revealed 1.365×10^{8}cfu/g and 4 different colonies were selected and further isolated to obtain pure isolate and were placed in slant bottles for proper preservation.

Lipase production on tributyrin agar medium

Out of the 4 isolates evaluated for lipase activity, isolate 1 showed clear zone on the tributyrin agar medium presented in Table 1. Plate 1 revealed clear halo showing lipase activity on tributyrin agar by one of the isolates TRL While plate 2 showed no clear zone on tributryin agar by one of the isolates EC.

Table 1: Screening for lipase production.

Isolate code	Clear halo on tributyrin medium(diameter in mm)
YR	-
WS	-
TRL	28 mm
EC	-

Keys: YR, WS, TRL, EC: isolate code, -: no clear zone

Plate : Clear halo showing lipase activity on tributyrin agar by one of the isolates TRL.

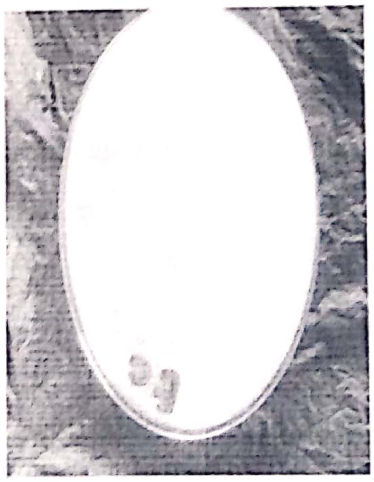

Plate 2: Showing no clear zone on tributyrin agar by one of the isolates EC

Identification of the lipase producer

The bacterial isolate which showed maximum lipase production was further characterized and identified by morphological and biochemical characteristics presented in Tables 2 and 3

respectively. The macro culture and biochemical characteristics of the lipase producing bacteria isolate is presented in Tables 2 and 3 below.

Table 2: Macro culture of the isolate that showed lipase activity on tributyrin agar medium

Characteristics	Margin	Elevation	Form	Texture	Pigmentation
Isolate	Lobate	flat	irregular	not mucoid	cream

Table 3: Biochemical tests results

Biochemical tests	Gr	Sh	Mt	Cat	Ox	Ct	Sth	Ch	Ma	Coa
Results	+ve	rod	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve

+ve:positive, -ve: negative, Gr: Grams reaction, Sh: Shape, Mt: Motility, Cat: Catalase, Ox: Oxidase, Ct: Citrate, Sth: Starch hydrolysis, Ch: Casein hydrolysis, Ma: Mannitol, Coa: Coagulase.

Effect of pH and Temperature on lipase activity

The effect of pH on activity of lipase produced by *Bacillus subtilis* is shown in Figure 1. The result showed that the pH increases, the lipase activity increases with optimum lipase activity (1.681µmol/mL/min) at pH 8.0. However, pH beyond 8.0, the activity of the enzyme decreases (Figure 1). *Bacillus subtilis* also showed optimum lipase activity (1.504µmol/mL/min) at 50°C (Figure 2), while decreased steadily at temperature beyond 50°C.

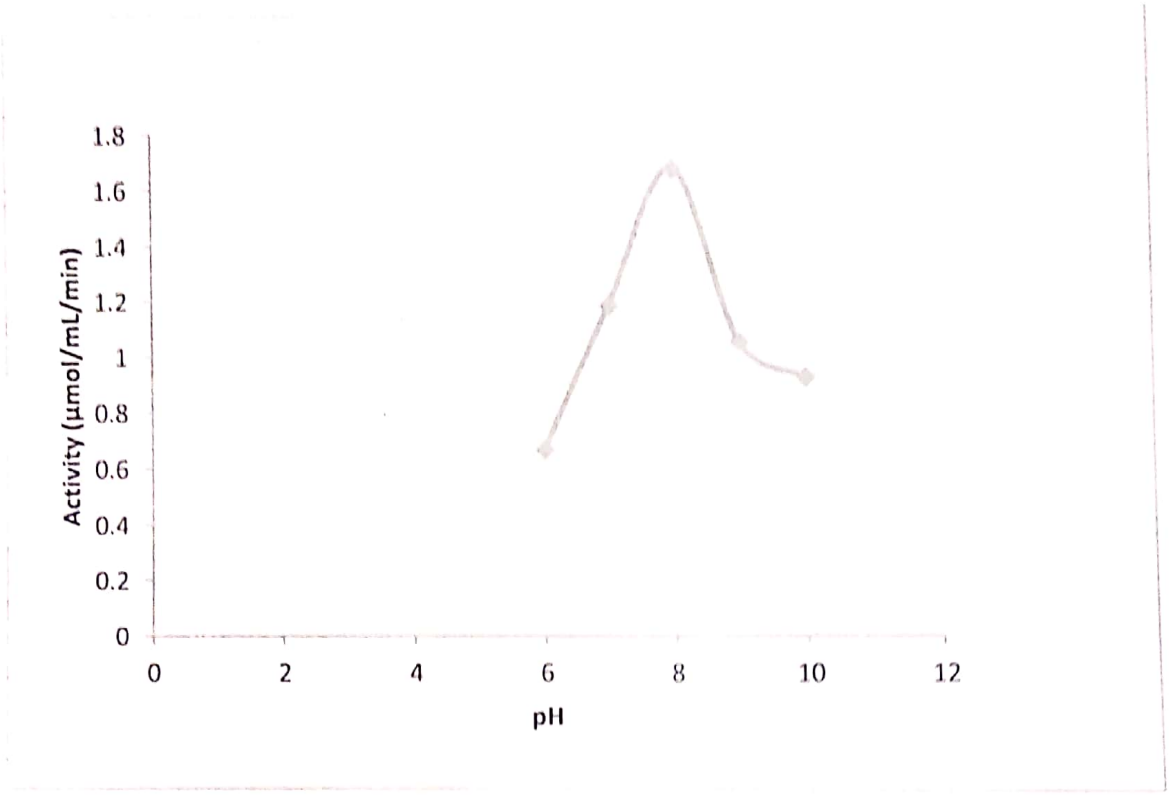

Figure 1: Effect of pH on the activity of lipase produced by *Bacillus subtilis*

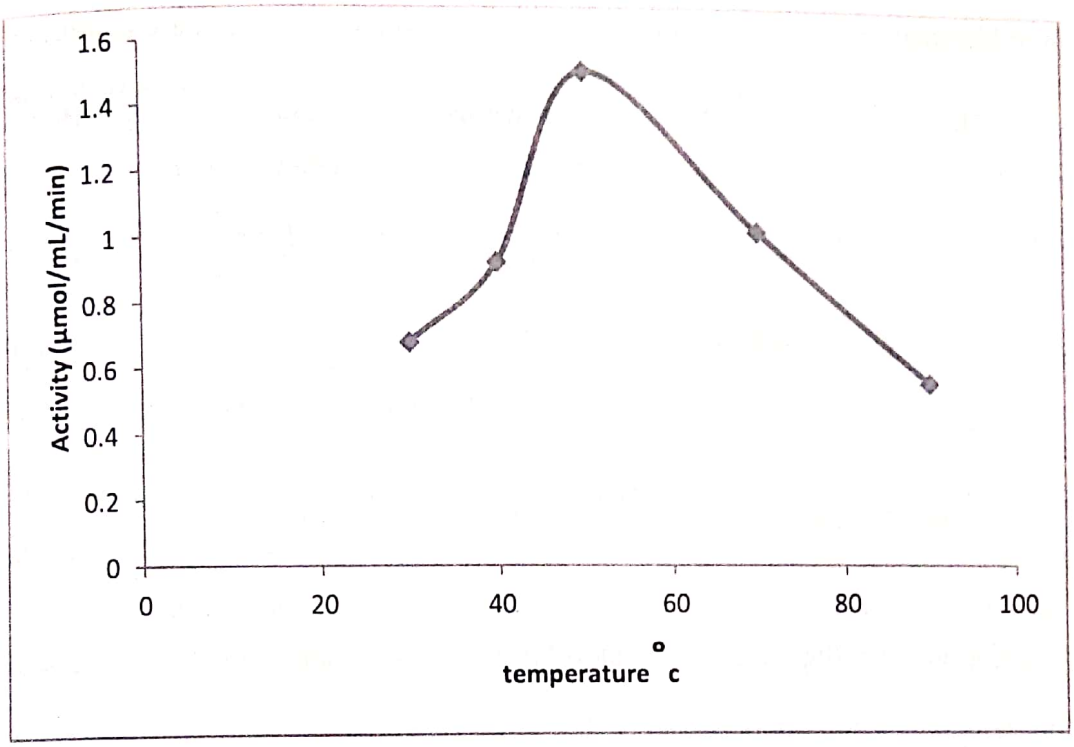

Figure 2: Effect of temperature on the activity of lipase produced by *Bacillus subtilis*

Regression Analysis on the lipase activity

The analysis evaluated the dependent variable (lipase activity) and the independent variables (pH and temperature) respectively to examine the effect of the independent variables on the lipase activity. Data was subjected to statistical analysis using regression analysis and analysis of variance (ANOVA) which showed that pH and temperature have significant effect on the activity of lipase from *P-value* of 0.043< 0.05 level of significance, which indicates that the regression model for checking the effect of pH on the lipase activity is significant(Table 4) and the *P-value* of 0.035< 0.05 level of significance indicates that the regression model for checking the effect of the temperature on the lipase activity is statistically significant (Table 5).

Table 4: Analysis of Variance for the Fitted Regression Model of the pH on the lipase activity

Source of variation	Df	Sum of squares	Mean square	f-value	p-value
Regression	1	0.079	0.079	0.479	0.043
Residuals	3	0.496	0.165		
Total	4	0.575			

R-Squared = 0.371, Adjusted R-squared = 0.138*Significant at p-value < 0.05 level of significance

Table 5: Analysis of Variance for the Fitted Regression Model of the temperature on the lipase activity

Source of variation	Df	Sum of squares	Mean square	f-value	p-value
Regression	1	0.035	0.035	0.317	0.035
Residuals	3	0.327	0.109		
Total	4	0.362			

R-Squared = 0.746, Adjusted R-squared = -0.206*Significant at p-value < 0.05 level of significance

DISCUSSION

Out of the four bacteria strains isolated in this study, *Bacillus subtilis* showed lipase production by clear halo on the screening medium. Similar results were observed where *Bacillus subtilis* strain from soil was optimally found to be active for lipase production (Bora and Kalita, 2007 and Veerapagu*et al.*, 2013). Bala *et al.* (2014), Bala (2016) and Bala *et al.* (2018) have reported

that *Bacillus subtilis* isolated from palm oil mill effluent (POME) revealed high lipase activity on solid media.

Effect of temperature and pH are essential for enzyme production as change in temperature could impact microbial growth and lipase activity. The temperature andpH affects stability of enzymes by changing the electrostatic interactions of their protein structure, producing changes in the amino acids' ionization status, which defines the secondary and tertiary structures of protein and therefore its activity and stability (Nema *et al.* 2019)

Initially, lipase activity increased with increase in the temperature and pH with maximum lipase activity observed at 50°C and pH of 8.0. On further increasing the temperature and pH, decrement in the lipase activity was observed. Another study reported that *Bacillus subtilis* possesses good temperature and pH stability (20-50°C; pH 4.0–8.0) during lipase production (Mukeshkumar *et al.*, 2012; Nema *et al.* 2019).Hence, optimum temperature and pH observed in the present study agrees with the range that was reported for lipase to retain its activity. Regression analysis revealed that temperature and pH were significant factor $p< 0.05$.

CONCLUSION

In this study, lipase producing *Bacillus subtilis* was isolated from soil. The optimum temperature and pH for the activity of the lipase were 50°C and 8.0, respectively. *Bacillus subtilis* which is a potential candidate for lipase production can be used for bioremediation of oil-contaminated wastewaters and soil as well as in industrial applications.

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