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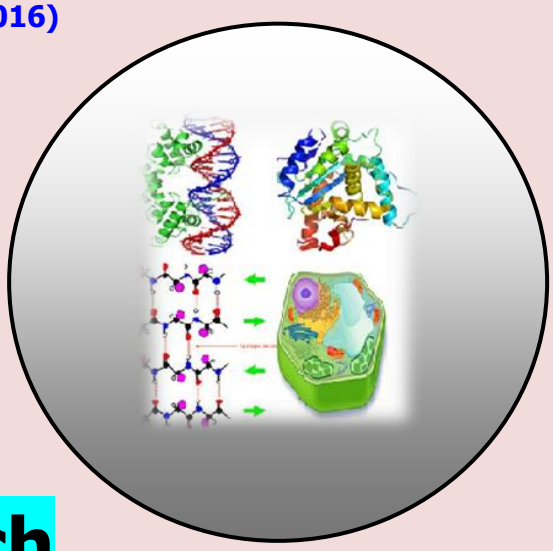
By

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RESEARCH PAPER

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Effect of pH and Temperature on the Activity of Lipase Produced by *Aspergillus niger* Cultured on *Citrus sinensis* Peel

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ABSTRACT

This study investigated effect of pH and temperature on the activity of lipase produced by Aspergillus niger during fermentation of Citrus sinensis peel. To obtain crude enzyme solutions, 100 ml of enriched media were mixed with 5 g of Citrus sinensis peel in 250 ml Erlenmeyer flask. Then, this mix was inoculated with 10 cfu/ml of microorganism and incubated at 35°C for 168 hours. After the seventh day of incubation, the crude enzyme was collected by filtration followed by centrifugation. The crude enzyme solutions obtained were subjected to ammonium sulphate precipitation, dialysis and gel filtration chromatography for further purification. The Michalis-Menten kinetic constants Km and Vmax for purified lipase were determined by varying concentrations of p-nitrophenyl laurate (p-NPL). Evaluation of effect of temperature and pH on purified enzyme activity was also carry out. This purification procedure resulted in 11.81 fold purification of lipase with 14.10% final yield. Sixteen (16) fractions of 5 ml each were collected after gel chromatography, however, only fraction 8 gave higher lipase activity. The values of Km and Vmax as calculated from the Lineweaver-Burke plot using p-nitrophenyl laurate (p-NPL) as substrate were 1.229 mg/ml and 8.097U/ml respectively. Maximum lipase activity of a purified lipase was observed in a wide range of temperature and pH values with optimum temperature of 39°C and pH 9.
Keywords: Aspergillus niger; Citrus sinensis; Fermentation; Lipase, Ph and Temperature.

INTRODUCTION

Lipase (EC. 3.1.1.3) is a generic name given to any enzyme that catalyzes the hydrolysis of fats (lipids) (Svendsen, 2000). Lipases are a subclass of the esterase that perform essential roles in the digestion, transport and processing of dietary lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms. Genes encoding lipases are even

present in certain viruses (Lepetit *et al.,* 2016; Wang *et al.,* 2016). The various applications of lipases have been reported in diary, textile, detergent, food processing industry (such as in fats and oil industries, bakery industries and dairy industries) as well as in medicine. This wide range of applications of lipase may be partly due to their substrate specificity such as fatty acid, alcohol, group- and stereo-specificity. The commercially available lipases are mainly obtained from microbes such as fungi and bacteria (Golani *et al*., 2016; Wongwatanapaiboon *et al*., 2016; Oliveira *et al*., 2017). The most industrious species of lipase producing microorganisms belongs to genera *Geotrichum, Penicillium, Aspergillus* and *Rhizomucor* (Khan *et al.,* 2016; Kumar *et al.,* 2016). Microorganisms have produced lipases that are extracellularly inducible in nature. These lipases are produced within the cell and transported to its external environment (Komnatnyy et al., 2014; Ekinci *et al*., 2016; Ali *et al*., 2017). Lipases synthesized by microbes constitute an important group of biotechnologically significant enzymes, mainly because of flexibility of their applied properties and ease of mass production (Dutta Banik *et al.,* 2016; Xie and Zang, 2016; Mutturi *et al*., 2017). The lipases produced by microorganisms have a wide range of applications in biotechnological field but the major hindrances against the inclusive use of lipase is the high cost of lipase production. However, agricultural wastes that contains high fats contents like *Citrus sinensis* peel can serve as an alternative for lipase production by optimizing some parameters like pH and temperature and to achieve the above objective, emphasis will be on production of lipase from *Citrus sinensis* peels, evaluation of the activity of the enzyme produced at different purification steps and studying the influence of pH and temperature on the enzymatic activity of the most active fraction(s).

MATERIAL AND METHODS

Agricultural waste (*Citrus sinensis* Peel)

The substrate (*Citrus sinesis* peel) was obtained from Malete Market, Malete, Moro Local Government, Kwara State, Nigeria. The substrates were air dried for 24 hrs. The dried samples containing 0.07 % moisture were then milled with Marlex Excellent Grinder (Mumbai, India) and sieved at the Biochemistry Laboratory of Kwara State University, Malete to make the substrate more susceptible for enzymatic degradation. The peel was authenticated and identified with voucher number UILH/001/996 at the Herbarium Unit of the Department of Plant Biology, University of Ilorin, Ilorin, Kwara State.

Microorganisms and Culture Conditions

Aspergillus niger was obtained from microbial culture bank, Microbiology Department, University of Ilorin, Ilorin, Nigeria. The microorganism was maintained at 4°C on slanted Potato dextrose agar (PDA). It was then sub-cultured on fresh agar plate and incubated for 72 hours. This was maintained at 4°C and used throughout the experiment.

Lipase Production by *Aspergillus niger*

Enriched media for lipase production was prepared by mixing 0.5% peptone, 0.1% KH_2PO_4, 0.3 % $MgSO_4.7H_2O$ and into 100 ml of sodium phosphate buffer (pH 7.5). The 100 ml enriched media were introduced into 250 ml Erlenmeyer flask and 5 g of pulverized *Citrus sinensis* peel were added and autoclaved for 15 minutes at 121°C and 15 minutes. It was cooled down to room temperature (25°C) and 10 cfu/ml of microorganism was inoculated into the flask in sterilized condition. Erlenmeyer flask containing all the chemicals was incubated at 35°C for 168 hours to ensure inoculums are in log phase. On the seventh day, the broths were taken out of the incubator, filtered using Whatman No. 1 filter paper and the filtrate was taken as the crude enzyme while the supernatant was discarded.

Purification of Lipase

Ammonium Sulphate Precipitation

Ammonium sulphate of 70% saturation was added to 20 ml of filtrate (Crude enzyme), Precipitation was allowed to take place for 2 hours followed by centrifugation at 4000 rpm for 15 minutes. The precipitate was dissolved in a minimal amount of phosphate buffer (pH 7.5).

Dialysis

Dialysis bag of about 5m long was cut and soaked in distilled water for 24 hours so as to open its two ends. Dialysis was setup and the precipitate obtained from salting out was loaded into dialysis bag, and dialyzed for 24 hours with buffer changes every three hours.

Gel Filtration chromatography

Column was loaded with gel (sephadex G-100) and left for 24 hours so as to be well packed, after which it was loaded with the dialyzed enzyme and buffer was added as a mobile phase. Fractions were collected from the column and protein content was measured spectrophotometrically at 280nm. Lipase activity of each fraction was assayed for and the fractions with highest activity were used for characterization of the enzyme.

Assay of Lipase Activity

Lipase activity was measured with spectrophotometric assay method with *p*-nitrophenyl laurate (*p*-NPL) as substrate. The reaction buffer with 12.5 mM *p*-NPL was prepared by dissolving substrate in acetonitrile with Tris-HCl buffer (pH 8.0) containing 10 mM CaCl2. Enzyme solution (0.2 ml) was added to the reaction buffer (0.8 ml). The progress of the reaction was monitored by the change of the absorbance at 410 nm over a 5 minutes' period at 45°C using a spectrophotometer. The molar extinction coefficient of *p*-nitrophenol (*p*-NP) (a = 1.5 × 104 cm2/mol). One enzyme unit (U) was defined as the lipase activity that liberated 1µmol equivalent of *p*-NP per minute under the standard assay conditions.

Determination of Protein Concentration

Concentration of protein was determined by following the method of Lowry *et al*. (1951) using Bovin Serum Albumin (BSA) as standard.

Effect of Temperature and pH on Purified Lipase

The purified enzyme was incubated with substrate in an incubator with temperatures set at 35, 36, 37, 38, 39 and 40°C after which lipase activity was determined. The effect of pH on lipase activity was studied by incubating the enzyme with p-nitrophenyl laurate, prepared with sodium phosphate buffer of pH 6, 6.5, 7, 7.5, 8, 8.5 and 9.

Effect of Substrate Concentration and Determination of Kinetics Parameter (K_m and V_{max})

The effect of varying concentrations of substrate on purified lipase was determined using p-nitrophenyllaurate as substrate. The apparent kinetic parameters (V_{max} and K_m) of the lipase were determined by varying the concentration of the p-nitrophenyllaurate from 2 mM to 10 mM.

Statistical Analysis

All experiments and enzyme assays were expressed as mean ± SEM of three replicates (n=3). Graphpad prism version 5.02 was used to plot all the graphs.

RESULTS

Purification Lipase

The purification summary and elution profile of the lipase are shown in Table 1 and Figure 1 respectively. The crude enzymes produced under optimum fermentation conditions were first precipitated by 70 % saturated $(NH_4)_2SO_4$ with a specific activity of 2.85 U/mg and 1.34fold purification. By gel filtration, the enzyme was highly purified to 11.81 fold with a yield of 14.10 % and a specific activity of 25.16 U/mg (Table 1). The elution profile diagram of lipase from *Aspergillus niger* revealed that fraction eight (8) gave maximum enzyme activity and it was later used for further studies (Figure 1). The Line weaver-Burk plot of purified lipase from *Aspergillus niger* and kinetic parameter values are shown in Figure 2 and Table 2 respectively.

Table 1. Purification summary of lipase produced from *Aspergillus niger* under optimum fermentation conditions.

Purification steps	Total Volume (ml)	Total Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude	100	5.53	2.60	2.13	100	1.00
$(NH_4)_2SO4$	20	1.48	0.52	2.85	26.76	1.34
Dialysis	6	1.38	0.12	11.31	24.95	5.30
Gel Filtration	5	0.78	0.03	25.16	14.10	11.81

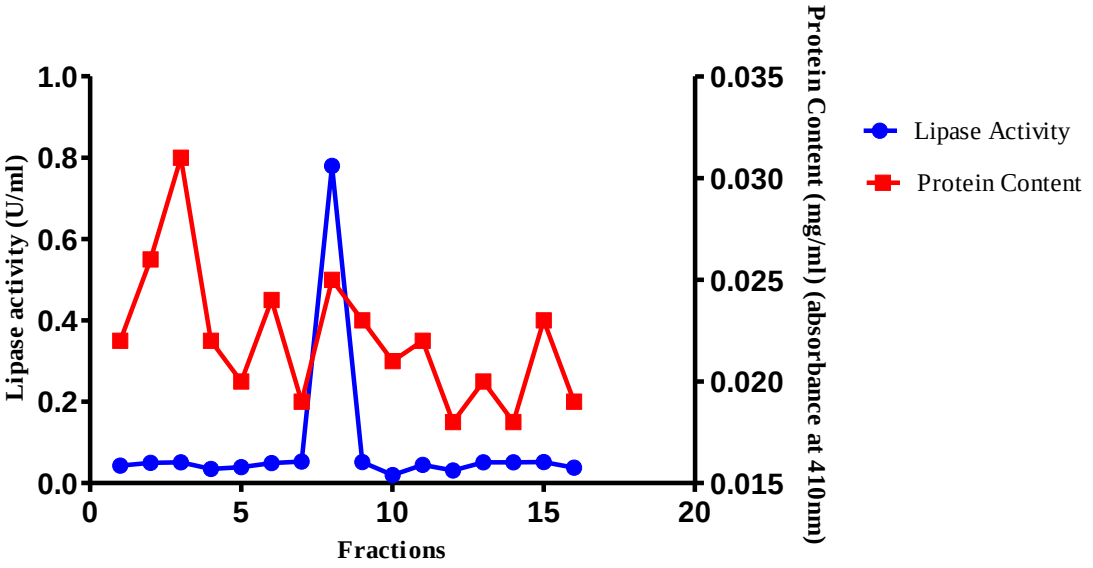


Figure 1. Elution profile of *Citrus sinensis* Peel lipase on sephadex G-100 chromatography.

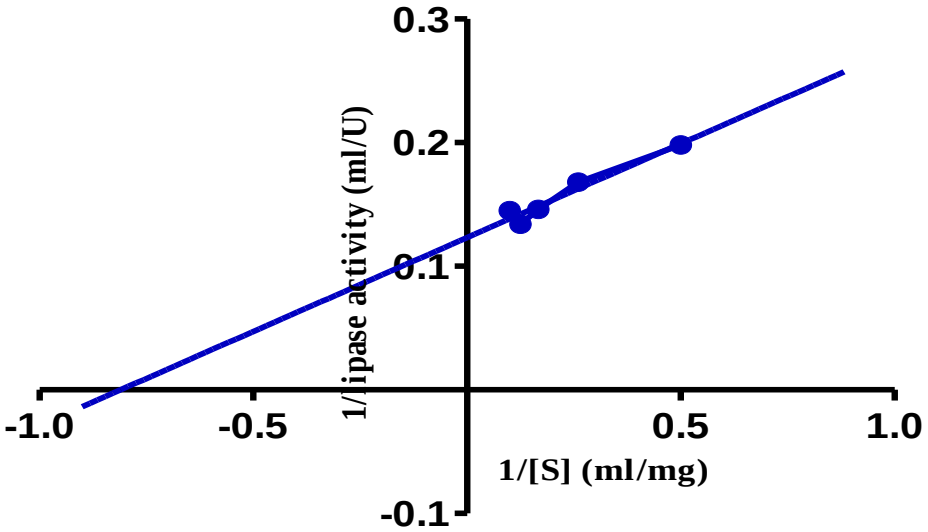


Figure 2. Line- weaver Burk plot of purified lipase (fraction 8) from *Aspergillus niger.*

Table 2. Kinetic properties of lipase (fraction 8) produced by *Aspergillus niger.*

Parameters/Substrate	*p*-nitrophenyl laurate (*p*-NPL)
K_m (mg/ml)	1.229
V_{max}(U/ml)	8.097

The values of K_m and V_{max} of the purified lipase (Table 2), as extrapolated from the Lineweaver–Burk plot, were 1.229 mg/ml and 8.097 U/ml respectively (Figure 2). This result showed that K_m value of the lipase (p-NPL as substrate) from *A. niger* has appreciably lower K_m which is an indicative that it has a strong affinity for *p*-nitrophenyllaurate. The optimum temperature of the enzyme was found to be 39°C (Figure 3) while the optimum pH of the enzyme was found to be around pH 9.0 (Figure 4).

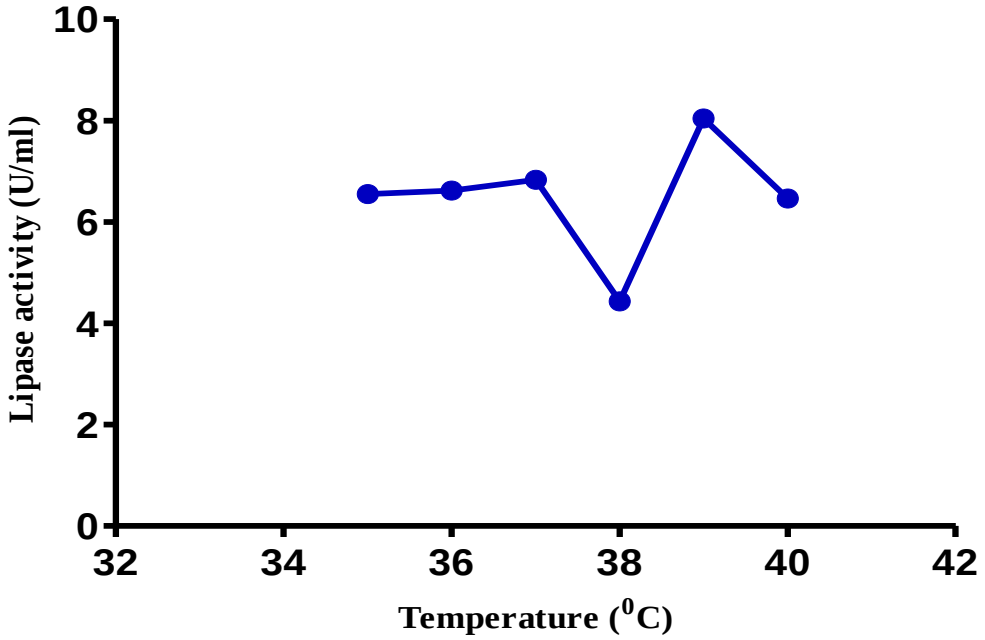


Figure 3. Effect of temperature on the activity of purified lipase (fraction 8).

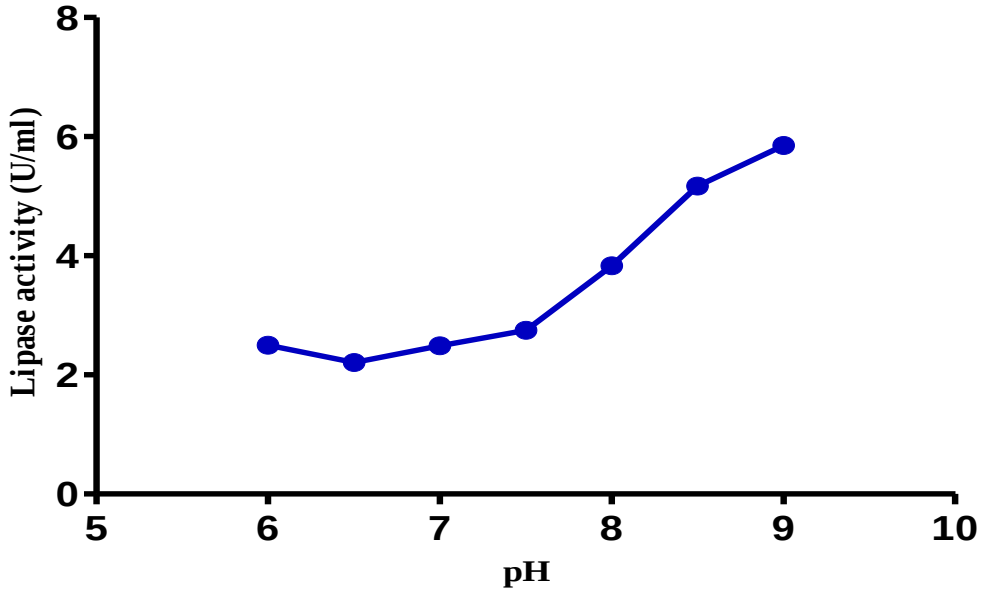


Figure 4. Effect of pH on the activity of purified lipase (fraction 8).

DISCUSSION

The growth of isolated *Aspergillus niger* in a medium containing *Citrus sinensis* peel led to the expression of large amount of lipase in the form of crude enzymes. Different enzymes are found in different partition in the cell and thus require specific purification procedures.

A very sharp reduction in the total activity of lipase following the purification from crude to gel filtration chromatography and an analogous increase in the specific activity of the same enzyme may be credited to the removal of large amount of inhibitors present in the crude enzymes. Also, a decrease in percentage yield of lipase and a corresponding increase in the purification fold may be attributed to denaturation of enzyme during purification steps. The michaelis-menten's constant, K_m, measures the affinity of enzyme for its substrate. The lower the K_m value of enzyme, the greater the affinity the enzyme will have for its substrate. This result obtained from this study showed that K_m value of the lipase (p-NPL as substrate) from *A. niger* has appreciably lower K_m which is an indicative that it has a strong affinity for p-nitrophenyllaurate. There was a gradual increase in the activity of purified lipase between the temperature of 35–37°C and maximum enzyme activity was obtained at 39°C (Figure 3). This result was similar to result of *Pseudomonas fluorescens* reported by Ganasen *et al*. (2016). The optimum temperature for lipase from *Rhizopus oryzae*, showed maximum activity at 39°C (López *et al*., 2016) which supports the present studies, the activity was decreased sharply at 38°C and 40°C. Microorganisms generally, have been shown to be more sensitive to the concentration of hydrogen ions present in the medium and pH has been shown to be very important factor which affects the growth and production of enzymes during fermentation (Edupuganti *et al*., 2017). Also, nature of enzymes implies that pH will affect the ionization state of the amino acids that confers the structures of the enzyme and hence, controls its overall activity. Each enzyme has its own optimum ranges for pH where it will be most active. The results obtained from this study revealed the highest lipase activity at pH of 9. This result was similar to the work carried out by Cai *et al.* (2016) who reported that purified lipase optimum pH from *Pseudomonas aeruginosa* ranges between pH of 9 and 10.

In conclusion, lipase produced from *Aspergillus niger* cultured on *Citrus sinensis* peel required an optimum temperature of 39°C and pH of 9 for maximum activity.

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