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Comparative Studies on the Kinetic Properties of Lipases Purified from *Aspergillus nidulans* and *Aspergillus niger* LC 269109

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Abstract

Lipases are enzymes with potential industrial applications but their supply is not sufficient for efficient utilization. The present work focused on lipase from two fungal isolates (*Aspergillus niger* LC 269109 and *Aspergillus nidulans*). The effects of pH, temperature and substrate concentration on lipase activity, as well as some kinetic parameters were determined. The lipase from *Aspergillus nidulans* was optimally active at pH 7 and temperature of 40°C, while the lipase from *Aspergillus niger* LC 269109 was found to be optimally active at pH 6 and temperature of 50°C. Lipase from *Aspergillus nidulans* was found to have a larger K_m (17.54 mg/ml) than the lipase from *Aspergillus niger* LC 269109 (9.71 mg/ml) and a higher V_{max} (769. 23 µmol/min) compared to that of lipase from *Aspergillus niger* LC 269109 (714.29 µmol/min). Hence, these isolates of *Aspergillus nidulans* as well as the *Aspergillus niger*, LC 269109 may be exploited as cheap source of lipase.

Keywords: Characterization; Biodiesel; Temperature; K_m and V_{max}

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Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a group of serine hydrolases which hydrolyze triacylglycerols [1,2]. These enzymes act at an oil-water interface, liberating free fatty acids diglycerides, monoglycerides, and glycerol [3]. The activity of lipases is affected by a number of factors as pH, temperature, substrate concentration, and substrate specificity [4-6].

Lipases are widely distributed in the biota: microbes, plants and animals which provide enzymes endowed with different substrate specificities, catalytic properties and robustness towards organic solvents [7-9], but microbial sources have more potentialities for industrial applications because of ease of genetic manipulations, absence of seasonal fluctuations, exponential growth of microbes, among others [10,11].

Owing to their versatility, broad specificity, and stability in different reaction media [12], lipases have been among the most used biocatalysts [13,14]. Applications of lipase cover a wide range of industries such as food processing, pharmaceutical, biofuel, oleo-chemical, detergent, pulp/paper, leather, textile industries; and in medical diagnostics [15-18].

Microbial lipases have been produced via SmF-submerged fermentation or SSF-solid state fermentation, using different microbial sources, under controlled/optimized culture conditions/parameters [19]. Several articles in the literature have reported isolation and characterization of lipases from different sources. Despite these efforts, the price of these enzymes in the world market still remains so high [20]. Considering the vast potentials lipases hold and the current high cost of the enzymes, this research was embarked upon with the aim of identifying cheaper sources of lipase. The present research reports comparative studies on the kinetic properties of lipases partially purified from two fungal isolates *A. nidulans* and *A. niger*.

Materials and Methods

Microbial materials

Pure isolates of *Apergillus niger* LC 269109 (*A. niger* LC 269109) and *Aspergillus nidulans (A. nidulans*) maintained on Agar slants were obtained from the department of Microbiology, University of Nigeria, Nsukka.

Reagents

Folin-ciocalteau phenol reagent was obtained from BDH, England;

β-mecapthoethanol was from LABO Chemie, India; while Bovine Serum Albumin was from Sigma-Aldrich, USA. All other reagents were of analytical grade, obtained from Sigma-Aldrich, USA.

Screening of microbes for lipase production

Screening the microbes (*A. niger* and *A. nidulans*) for lipolytic activity was done using the method reported by Narasimhan and Valentin [21], with slight modification. Tween 80: Agar plates were prepared with a medium containing peptone 10 g, Agar-Agar 1.5 g, NaCl 0.5 g, $CaCl_2$ 0.01 g, and 1 ml of Tween 80. After solidification, the plates were streaked with fungi and observed for zones of precipitation at 37°C for 48 h.

Production and partial purification of lipase

Lipase was produced using submerged fermentation with medium consisting of peptone 3% (w/v), $MgSO_4.7H_2O$ 0.5% (w/v), KH_2PO_4 0.2% (w/v), KCl 0.05% (w/v), and olive oil 1% (w/v). The fermentation broth was filtered through 4 layers of muslin cloth under ice and the filtrates centrifuged at 5000 x g for 15 minutes at 4°C. The supernatant was collected as crude enzyme and stored at 4°C for further studies **(Tables 1 and 2).**

Crude lipase was subjected to ammonium sulpahte precipitation at 70% saturation for 36 h at 4°C. Lipase precipitates obtained by centrifugation at 5000 rpm for 20 minutes were resuspended in 30 ml sodium phosphate buffer (150 mM, pH 6.5), dialysed at 4°C against the same buffer overnight, and stored at 4°C for further use.

Lipase assay and estimation of protein concentration

Lipase activity was assayed using the colorimetric method of Duncombe et al. [22]. Olive oil emulsion was prepared by homogenizing 2 g, each, of Arabic gum and olive oil in phosphate buffer (150 mM, pH 7). Assay mixture contained 5 ml of olive oil emulsion, 5 ml of sodium phosphate buffer (pH 7) and 1 ml of enzyme, all incubated at 37°C for 30 minutes. Reaction was stopped by adding 1 ml of 6 N HCl and copper soaps of FFAs formed by adding 2.5 ml of copper reagent (consisting of 10 volumes of 6.45% $Cu(NO_3)_2$, 9 volumes of 1M triethanalomine, and 1 volume of 1N acetic acid). The copper soaps of FFAs were extracted using chloroform and quantified at 440 nm using sodium diethyldithiocarbamate as a colour reagent. One unit of lipase activity was defined as 1 µmole of free fatty acids released per minute under the assay conditions.

Protein concentration of soluble enzyme preparation was quantified by method of Lowry et al. [23], using Bovine Serum Albumin (BSA) as a standard.

Characterization of Enzymes

Effect of pH and temperature

The effect of pH on lipase activity was studied by determining lipase activity at different pH values ranging from 3 to 9 at interval of 1. The buffer systems used were: sodium acetate buffer (pH 3-6), sodium phosphate (pH 7), Tris-HCl (pH 8-9). The effect of temperature on lipase activity was investigated by carrying out lipase assay at a temperature range of 20°C to 70°C at interval of 10°C.

Enzyme kinetics

Lipase was assayed in reaction buffer (pH 7, being the optimal value) at 37°C with different concentrations (10, 30, 50, 70, 90, and 110 mg/ml) of olive oil emulsion. The V_{max} and K_m were then calculated from Lineweaver-Burk (double reciprocal) plot **(Figures 1 and 2).**

Results and Discussion

Screening of fungal isolates for lipase production

The first step of the work involved screening of the fungal isolates for lipase production. **Figure 3** shows photographs of cultures of the fungi on Tween 80: Agar medium after incubation for three days at room temperature. Whitish zones of precipitation were noticeable after three days and the intensity remained until day five.

Table 1 Purification table for *A. nidulans* lipase.

Purification Step	Volume of Enzyme Protein (ml)	Protein Concentration (mg/ml)	Lipase Activity (U/ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold
Crude Enzyme	500	3.31	227.47	1653.15	113736.85	68.80	1
$(NH_4)_2SO_4$ Precipitation (70%)	30	1.31	197.72	39.36	5931.57	150.69	2.19
Dialysis	40	0.43	232.63	17.36	9305.26	536.14	3.56

Table 2 Purification table for *A. niger* lipase.

Purification Step	Volume of Enzyme Protein (ml)	Protein Concentration (mg/ml)	Lipase Activity (U/ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold
Crude Enzyme	500	3.31	178.60	1653.15	89298.25	54.02	1
$(NH_4)_2SO_4$ Precipitation (70%)	30	1.46	135.61	43.85	4068.42	92.78	1.72
Dialysis	40	0.62	225.44	24.96	9017.54	361.22	3.89

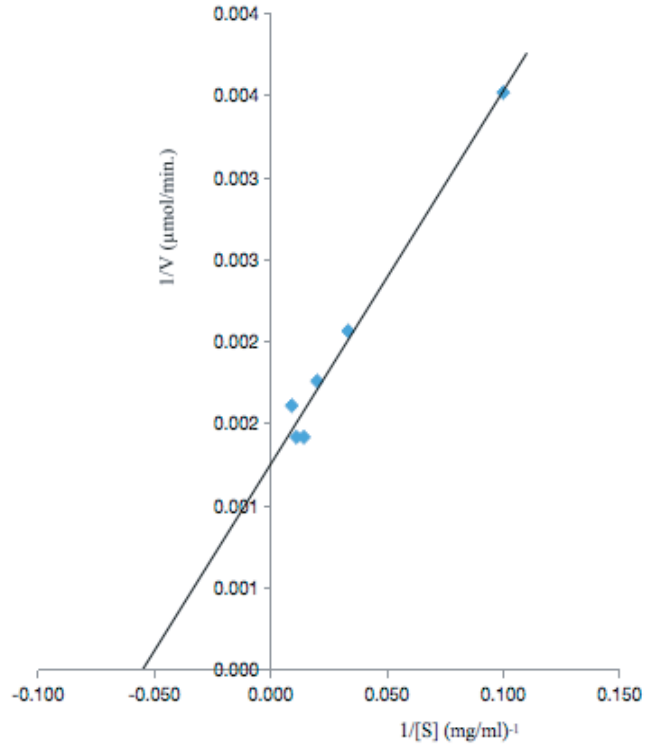

Figure 1 Lineweaver-Burk plot for *Aspergillus nidulans* lipase.

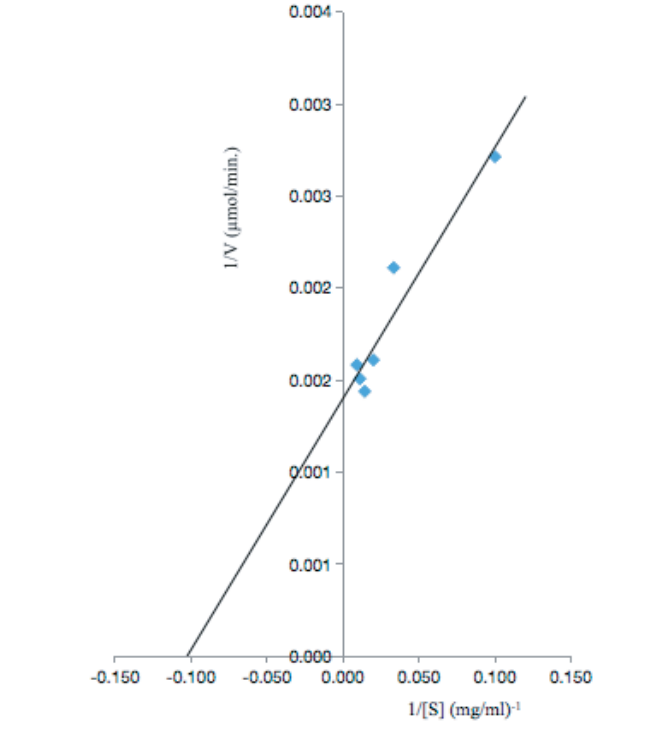

Figure 2 Lineweaver-Burk plot for *Aspergillus niger* lipase.

This indicates the ability of the fungi to produce extracellular lipase and similar cases have been reported [21]. As the lipase is excreted into the medium, it hydrolyses Tween to release esterified long-chain fatty acids that then react with calcium ions (Ca^{2+}) to form insoluble salts (precipitates) that are responsible for the white colour of the zone of precipitation [24].

Characterization of lipase

Effect of pH on activity of lipase: The results obtained from pH studies revealed that the lipase from *A. nidulans* had optimum pH of 7, whereas *A. niger* LC 269109 lipase was found to have a pH optimum of 6. Enzymes are most active at their pH optima, since that is when their active sites have maximum interaction with the substrate. Any drastic change in the pH of a medium, therefore, leads to denaturation of the enzyme, resulting in the loss of its activity, hence the interest in studying enzymes with respect to pH **(Figure 4).**

Different investigators have reported similar findings regarding variation of lipase activity with pH. Das et al. reported pH 4 as

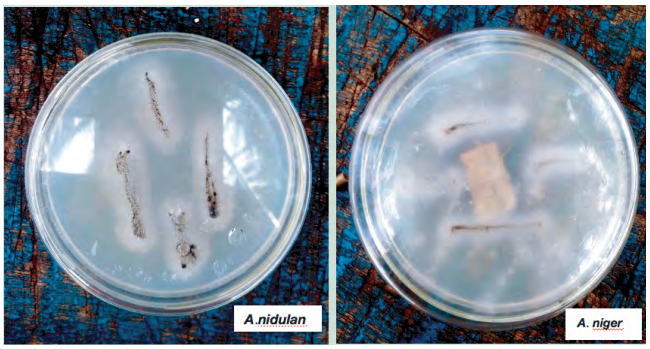

Figure 3 *Aspergillus* (Left = *nidulans*, Right = *niger*) on Tween 80: Agar plates.

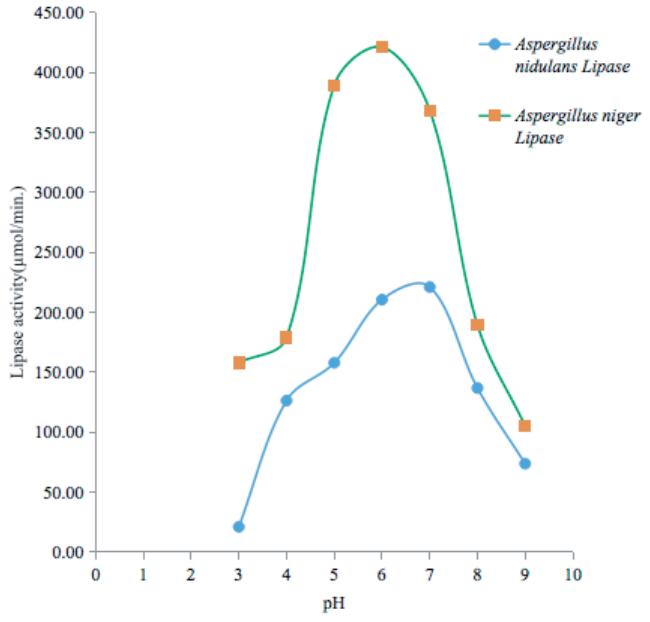

Figure 4 Effect of pH on activity of lipase from *Aspergillus nidulans* and *Aspergillus niger.*

the optimal pH for lipase activity of *A. tamarii*, Sethi et al. [25] reported pH optimum of 6 for lipase from *Aspergillus terreus* [26] also reported pH 6 for lipase from another strain of *Aspergillus terreus* [27], however, reported a pH optimum range of 7.3 - 8.2 for lipase from *Candida zeylnoides.* While Adham and Ahmed [28], reported pH 7.2 for a lipase from *Aspergillus niger* NRRL3 and in the case of Kareem et al. [4], pH 7.0 was the optimal pH for lipase from *Aspergillus flavus* PW2961.

Change in pH might influence the structure of substrates or the active site 'lid' so as to determine substrate binding [29].

Effect of temperature on activity of lipase: Lipases activity also varied with temperature. Lipase activity from *A. nidulans* increased to a maximum at 40°C while activity of lipase from *A. niger* LC 269109 reach maximum at 50°C. Beyond these values, activity dropped in each case **(Figure 5).** The lipase from *A. nidulans* retained up to 70% of its activity at 30°C for 30 minutes whereas the lipase from *A. niger* LC 269109 had up to 62% of its activity at 60°C for 30 minutes. These results obtained in the present research bear concordance with existing literature. Yan et al. reported a lipase from *Rhizomucor endophyticus* with an optimum temperature of 40°C; Sethi et al. reported a lipase from *Aspergillus terreus* NCFT 4269.10 with 50°C as its optimum temperature, Liu et al. [30] found 50°C as optimum for *Aspergillus niger* AN0512 lipase, whereas Lanka et al. [31], Mahmoud et al. [26], and Khan et al. [32] all reported different (fungal) lipases with 30°C as their optimal temperature. Lipases with other temperature optima were reported by Das et al. 37°C,

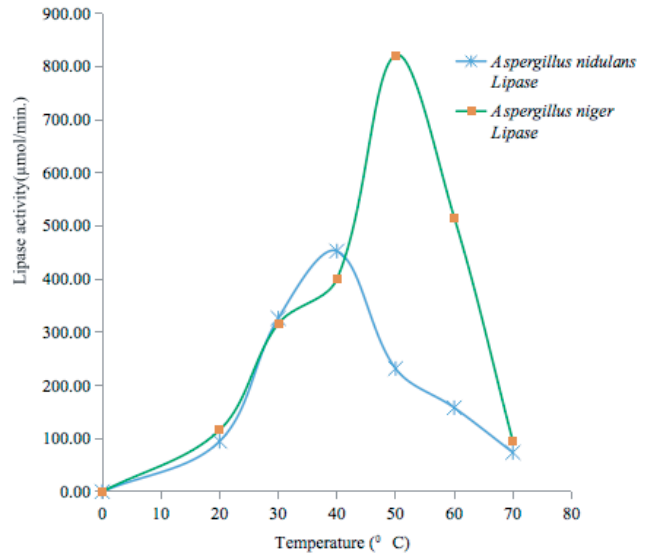

Figure 5 Effect of temperature on activity of lipase from *Aspergillus nidulans* and *Aspergillus niger.*

Table 3 Kinetic parameters of *A. nidulans* Lipase and *A. niger* lipase.

Lipase Source	V_{max} (µmol/min)	K_M (mg/ml)
Apergillus nidulans	769.23	17.54
Apergillus niger	714.29	9.71

Aspergillus tamarii JGIF06; Bharti et al. [33]: 40°C, *Aspergillus japonicas*; Kareem et al. 45°C [4], *Aspergillus flavus* PW2961 [28]: 60°C, *Aspergillus niger* NRRL3. In the case of Adham et al. the lipase retained over 90% of its activity even at 70°C. Variation in temperature may affect enzyme-substrate interaction by altering the enzymes' three-dimensional structures or the kinetic energy of colliding reactants.

Determination of kinetic parameters

V_{max} and K_m, were calculated from the double reciprocal plots for both of the two lipase sources. Lipase from *A. nidulans* had a higher V_{max} (769.23 µmole/min) than the lipase from *A. niger* LC 269109 (714.29 µmole/min) and a larger K_m (17.54 mg/ml) than the lipase from *A. niger* LC 269109 (9.71 mg/ml). Das et al. reported a lipase from *A. tamarii* JGIF06 with K_m and V_{max} of 330.4 mg and 53,690 U/ml/min, respectively. Pathak et al. [34] found K_m values ranging between 1.0 to 4.5 mg/ml and a V_{max} range of 34 to 187 U/mg for different microbial lipases. The V_{max} and K_m values (using olive oil as substrate) for yet another microbial lipase were found to be 35.34 mM/min and 312.98 mM, respectively [35]. Compared to *A. nidulans* lipase, *A. niger* LC 269109 lipase expressed a lower K_m value in the present study. This indicates that the A. *niger* LC 269109 lipase requires a smaller quantity of olive oil than the *A. nidulans* lipase requires for its saturation. Stated otherwise, *A. niger* LC 269109 lipase was found to have more affinity (or be more specific) for olive oil than the A. *nidulans* counterpart [36]. The magnitude of K_m varies widely with the identity of the enzyme and the nature of the substrate and is also a function of temperature and pH. From the V_{max} values, however, it could be seen that the *A. nidulans* lipase converted more substrates to products per minute than the *A. niger* LC 269109 lipase **(Table 3).**

Conclusion

The fungal isolates (*A. niger* LC 269109 and *A. nidulans*) are capable of producing lipase. The enzymes from the two sources were purified up to 4-folds. Maximal enzyme activity was found to be (under the assay conditions): pH 7, 40°C, 70 mg/ml of substrate (in the case of *A. nidulans* lipase) and pH 6, 50°C, 70 mg/ml of substrate (in the case of *A. niger* LC 269109 lipase). The K_m for *A. nidulans* lipase was found to be higher than that of the *A. niger* LC 269109 counterpart whereas the reverse was the case in terms of V_{max}. Thus, the two isolates are cheap sources of lipase for biotechnological applications.

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