Nig. J. Biotech. Vol. 32 (2017) 77 - 82
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org
DOI: http://dx.doi.org/10.4314/njb.v32i1.11

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Purification and Characterization of Lipase from *Aspergillus flavus* PW2961 using Magnetic Nanoparticles.

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(Received: 08:03:2017; Accepted: 05:04:2017)

Abstract

Lipase from *Aspergillus flavus* was purified in a single step purification using $MnFeO_4$ magnetic nano particles to achieve a 20.53- fold purification with specific activity of 11.29 U/mg and a 59% recovery yield. SDS-PAGE of lipase showed a single pure band with corresponding molecular weight of 35 kDa. The optimal temperature and pH for the enzyme activity were 45°C and 7.0 respectively. Addition of olive oil (1 %w/v) enhanced pH stability of the lipase with 86% residual activity at pH 7.0 after 6 h of incubation while the lipase was thermostable with 79% residual activity after 4 h of incubation. The enzyme activity was enhanced by Ca^{2+}, Mg^{2+} and Mn^{2+} while the presence of Cu^{2+}, Co^{2+} and Zn^{2+} exhibited inhibitory effect on the enzyme. The study therefore presented lipase from *Aspergillus flavus* PW2961 with potential in industrial and biotechnological application.

Key words: Lipase, purification, *Aspergillus flavus* PW2961, magnetic nanoparticles.
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Introduction

Lipases (EC 3.1.1.3) are enzymes capable of catalyzing the hydrolysis of fatty acids and glycerol into its monomeric units (Sharma et. al., 2001). Lipases are produced by many microorganisms and higher eukaryotes (Kamimura et. al., 2001). The ease with which enzymes could be isolated from microbes has made both bacteria and fungi predominant sources of lipase. However, fungi has been found to be the best lipase producers and are preferably used for various industrial purposes (Mahadik et. al., 2002). The most prospective lipase producers are found in the genera of *Aspergillus, Rhizopus, Penicillium* and *Trichoderma* (Kashmiri et. al., 2006).

Enzyme-catalysed reactions usually take place under relatively mild conditions, which make them ideal alternatives to various traditional chemical reactions. However, free enzymes usually have poor stability towards pH, heat or other factors and are difficult to recover and reuse (Kim et. al., 2006). Therefore, there is a great demand for ways of improving enzyme stability. Purification techniques, as a very powerful tool, have been intensively utilized to prepare various high-performance and economically-feasible biocatalysts with improved stability. Purification of enzymes using ammonium sulphate precipitation requires a lengthy separation period of about 12- 16 h for product recovery which often results in protein denaturation while the use of gel chromatography is slow and costly for developing economies (Kareem and Akpan, 2003).

There is a significant research interest in magnetic nanoparticles, as they offer a large spectrum of applications arising from their thermal, chemical, electrical, and magnetic field properties (Gu et. al., 2006, Wang et. al., 2007).

Magnetic nanoparticles are known to have multiple applications in the field of separation and purification technology (Yavuz et. al., 2009). One of the most important characteristics of these nanoparticles is to have a large volume of surface area (the smaller the size of the particle the larger the surface area) which leads to high binding capacity for the ligands. (Dios and Díaz-García, 2010). In this paper, lipase from *Aspergillus flavus* was purified using magnetic nano-particles (MNPs).

Materials and Methods

Microrganism

Strains of *Aspergillus flavus* were obtained from Culture Collection Unit of the Department of Microbiology, Federal University of Agriculture, Abeokuta, Nigeria. The isolates were sub-cultured on Potato Dextrose Agar (PDA) plates and preserved on PDA slant at 4^{o}C temperature (Balogun and Fagade, 2010).

Preparation of spore suspension

Spore suspension of *Aspergillus flavus* was prepared by scrapping 1 g of the spore from the petri dish into 10 ml of sterile distilled water (Kareem and Akpan, 2003) and then filtered using a sterile filter paper (Whatman, 125 mm). The filtrate was used as source of inoculum for the solid state fermentation.

Screening for lipase activity

Phenol red agar plates were prepared using phenol red (0.01% w/v) along with 1% (v/v) olive oil, 0.1% (w/v) $CaCl_2$, 2% (w/v) agar, two drops of tween-20 and the pH was adjusted to 7.2 (Singh et. al., 2006). A loopful spore of *Aspergillus flavus* was inoculated on the agar plates and incubated for 28^{o}C for 48 h. A change in color of phenol red from red to orange was used as an indicator of the lipase activity.

Production of Lipase

Production medium contained the following in percentage weight volume: rice bran 2.0, peptone 0.2, NH_4H_2PO4 0.1, NaCl 0.25, $MgSO_4 \bullet 7H_2O$ 0.04, $CaCl_2.2H_2O$ 0.04, olive oil 2.0 (v/v) and 1-2 drops Tween 80 as emulsifier. The medium was adjusted to pH 7.0. After 72 h of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4ºC and the cell free culture supernatant was used as the source of lipase.

Extraction of Lipase

Lipase extraction was carried out using 2.5 ml of 0.1 mM sodium phosphate buffer (pH 7.2) per gram of fermented medium (Couri et al., 2000). Crude enzyme extract was filtered using filter paper (Whatman, 125 mm), centrifuged at 8000 rpm for 20 min and the enzyme activity was determined.

Determination of lipase activity

Lipase activity was determined according to the method described by Sadasivam and Manikam (1996). A 250 ml Erlenmeyer flask containing 2 ml of 0.1 M phosphorus buffer, 1 ml of olive oil and 1 ml of crude enzyme extract was incubated at 40ºC for 30 min. The reaction was stopped by the addition of 5 ml of ethanol and then titrated against 0.1 N NaOH using phenolphthalein as indicator. Appearance of pale pink color indicated the end point.

Purification of lipase using magnetic nano - particles (MNPS)

Purification of the enzyme (protein) was carried out using magnetic $MnFeO_4$ nanoparticles MNPS (0.2 mg size) as described by the method of Okoli et al., (2011). The magnetic $MnFeO_4$ nanoparticles were washed three times each with 10 $mmolL^{-1}$ ammonium acetate buffer, pH 6.7, to equilibrate the particles and then suspended in buffer. The MNPS was transferred into crude lipase solution (10 ml) and incubated at 25°C in an orbitary shaker for 60 min. The unbound protein was separated by applying an external magnetic field and regarded as purified enzyme. The bound protein was eluted with 0.8 M NaCl in ammonium acetate buffer.

Protein determination

Protein content was evaluated by the method of Lowry et. al. (1951) using bovine serum albumin as standard. All measurements were performed three times and the average value was taken.

Effect of temperature on lipase activity

Effect of temperature on lipase activity was determined between 25°C and 80°C using olive oil (1% w/v) in 0.1 M sodium phosphate buffer (pH 7.2) as the substrate.

Effect of pH on lipase activity

Effect of pH on enzyme activity was evaluated by carrying out the reactions in different buffers in 0.1 M sodium acetate

buffer (pH 5- 5.5), 0.1 M sodium phosphate buffer (pH 6.0 -7.5) and 0. 1 M tris-glycerine buffer (pH 8-9) at 60°C for 1 h.

Enzyme stability profile

The thermostability of the purified lipase was determined by pre-incubating a solution of enzyme (5 ml) for 6 h at various temperatures (25-80°C), with or without olive oil (1 % w/v). pH stability of the purified lipase was determined by mixing the enzyme solution with sodium phosphate buffer (0.1 M) in a pH range (5-9) and kept at 30°C for 6 h. The residual enzyme activity was determined at 30 min regular intervals.

Effect of activators and inhibitors

Effect of various metal ions (Mn^{+2}, Co^{+2}, Ca^{+2}, Cu^{+}, Zn^{+2} and Mg^{+2}) and 5 mM ethylene diamine tetra acetic acid (EDTA) on enzyme activity was investigated on the purified lipase enzyme. A mixture containing 0.5 ml of the metal ion solutions and 0.5 ml of enzyme solution was incubated at pH 7.2 for 30 min at room temperature. The relative enzyme activity was measured under standard assay.

Molecular weight determination using SDS-page analysis

The molecular weight of the enzyme (crude and purified extract) was determined in a 10% SDS PAGE mini gels stained with Coomassie brilliant blue to visualize the protein (Okoli, 2011).

Results and Discussion

Purification of crude lipase resulted in a 20.5- fold purification with a final yield of 59% of total lipase in a single-step purification procedure as shown in Table 1. This purification fold is higher than previous report on a single step technique (Kareem and Akpan, 2003).

The result presented in Figure 1 indicated that the enzyme was active over a broad pH range (5.0- 9.0) with optimum activity at pH 7.0. This pH range was reported to offer wide industrial application (Romero et. al*.,* 2007). This findings is similar to that reported by Kamini et. al., (1998) for lipase activity from *Aspergillus niger* MTCC 2594.

Lipase from fungi has been exploited as valuable industrial enzyme due to its stability profile (Gulati et. al., 2000). The effect of pH on the stability of lipase showed that the enzyme was more stable at pH values 7.0 - 8.0 while retaining substantial enzyme activities of 86% and 80% at pH values 7.0 and 8.0 respectively after 6 h of incubation (Fig.3). Most microbial lipases are stable in the pH range of 2 to 10.5 (Falony et. al., 2006). Similar results have been reported for other fungal lipases (Salleh et. al., 1996; Falony et. al., 2006). The result showed that lipase activity when incubated at pH 9 for 1 h, retained 80% relative activity suggesting its potential use in various biotechnological processes especially in detergency.

The optimum temperature of lipase from *Aspergillus flavus* from this findings is shown to be 45°C (Fig. 2), this is in agreement with the study of Thamaraichelvan et. al. (2010), who reported 45°C as the optimum temperature of lipase from *Aspergillus niger.* The enzyme was thermostable in the temperature range of 40 to 60°C (Figure 4) by retaining 91%, 86% and 79% residual activity respectively after 4 h incubation, these characteristics indicated that the lipase is a thermostable lipase. Thamaraichelvan et. al., (2010) showed that lipase from *Aspergillus flavus* exhibited maximum thermostability between 40°C and 60°C when incubated for 1 hr.

Most fungal lipases have low thermal stability and are unstable at temperatures above 40°C (Thamaraichelvan et. al., 2010). The enzyme reported in this study was found to be completely stable at 50°C after 1 h. At 60°C, the enzyme maintained 81% stability 1 h after the initial activity. The thermostable lipase will offer advantages in various biotechnological processes such as detergency, trans-esterification, wastewater treatment (Denise et. al., 2015). Among the metal ions tested, Ca^{2+} and Mg^{2+} enhanced lipase activity with 139% and 124% relative activity respectively when compared to the control (Figure 5). These ions have been reported as cofactor for lipase (Dong et. al., 1999). The results obtained from this study were in agreement with the report of Amal et. al. (2016) that lipase from *Rhizopus oligosporus* was activated by Mn^{2+} Ca^{2+} and Mg^{2+}. The hydrolytic activity of the enzyme was inhibited by heavy metals such as Zn^{2+}, Co^{2+} and Cu^{2+} with 60%, 69% and 75% relative activity respectively, which suggests that metal ions may be interfering with enzyme activity by acting directly on enzyme active site (Supakdamrongkul et. al., 2010).

The purified lipase exhibited a single protein band with a molecular weight of 35 kDa (Figure 6). Similarly, lipase isoenzyme from an *Aspergillus niger* purified in a simple

adsorption step with C8 modified magnetic particles was reported to show a single pure band with a molecular weight of 35 KDa (Hernández-García et. al., 2014).

In conclusion, this study presented the potential of magnetic nanoparticles in the purification of crude lipase as a rapid, simple and efficient absorbent. The purified lipase produced by *Aspergillus flavus* was stable at alkaline pH and high temperature, therefore making it a potential candidate for industrial purposes.

Table 1: Summary of Purification of lipase from *Aspergillus flavus* PW2961

Purification Step	Lipase activity (U/ml)	Protein conc. (mg/ml)	
Crude lipase	2.75	5.02	0.5
$MnFeO_4$	1.92	0.17	11.

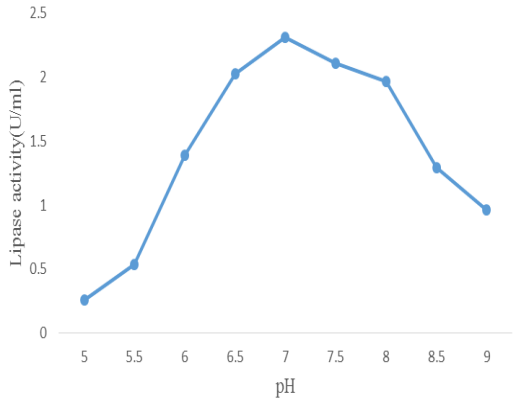

Figure 1: Effect of pH on lipase activity isolated from *Aspergillus flavus*

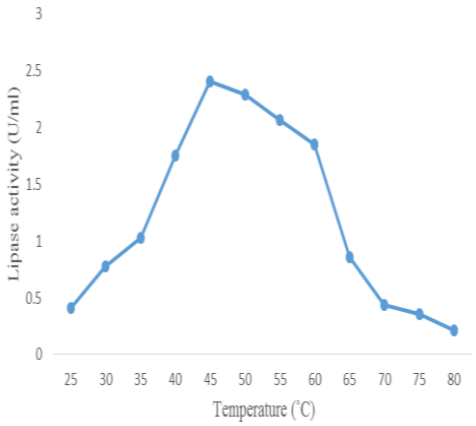

Figure 2: Effect of assay temperature of lipase activity from *Aspergillus flavus* PW2961

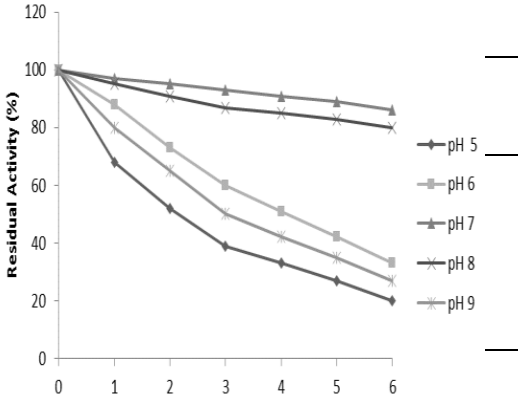

Figure 3: pH stability profile of lipase activity

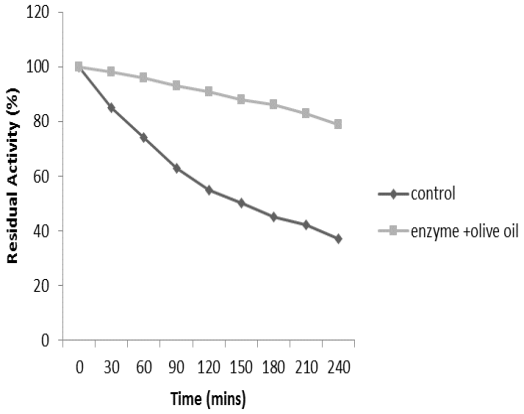

Figure 4: Thermostability profile of lipase activity from *Aspergillus flavus*

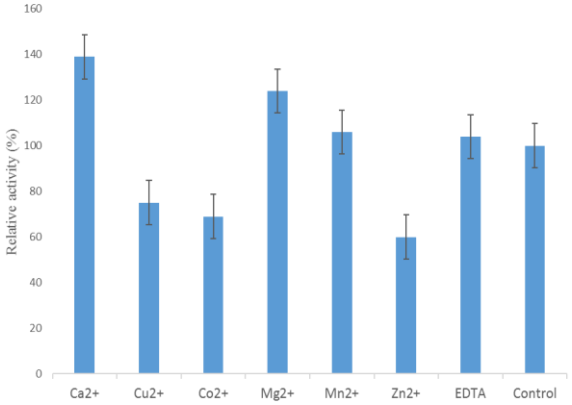

Figure 5: Effect of activators and inhibitors on purified lipase

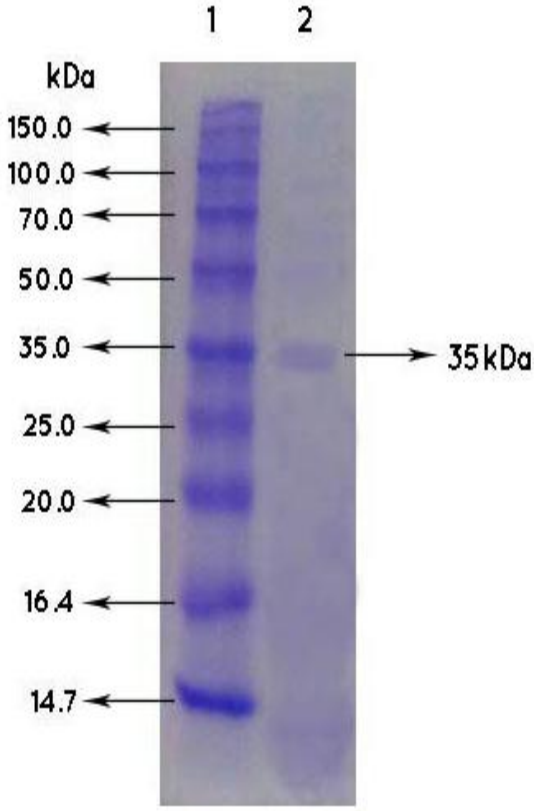

Figure 6: SDS-PAGE analysis of lipase from *Aspergillus flavus* PW2961. Lane 1-indicates molecular weight standard markers and Lane 2-shows purified lipase band of molecular weight approximately 35 kDa.

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