

ORIGINAL RESEARCH PAPER

LIPASE PRODUCTION FROM *MUCOR* SP. B6 ISOLATED FROM COCOA PROCESSING PLANT EFFLUENT IN ILE OLUJI, ONDO STATE, NIGERIA

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Abstract: The lipolytic activities of fungal isolates from a cocoa processing plant effluent were assessed both qualitatively and quantitatively. The fungus with the most appreciable lipolytic activity was selected and characterized morphologically. The optimum parameters for lipase production by the fungus were determined by studying the influence of carbon and nitrogen sources, pH, temperature, and incubation period, on production. Six fungal strains from the cocoa processing plant effluent which showed lipolytic activity were screened. The fungus with the most appreciable lipolytic activity was identified as *Mucor* sp. B6. Maximum enzyme production was obtained when the medium was incubated for 168 h, at a temperature of 30 °C, and pH 6.0. Olive oil and yeast extract were observed to be the most suitable carbon and nitrogen sources, respectively. The results obtained revealed that the fungal isolate could be a promising organism for the large-scale production of the industrially important lipase.

Keywords: effluent, enzyme, lipase production, lipolytic activity, *Mucor* sp. B6, olive oil, optimization

INTRODUCTION

Lipases (EC 3.1.1.3) (triacylglycerol acyl hydrolases) catalyze the hydrolysis of insoluble triacylglycerols releasing diacylglycerols, monoacylglycerols, and free fatty acids over aqueous and organic interface [1, 2]. They belong to the serine hydrolase family and mostly do not require any cofactor to catalyze the reactions [3]. Apart from hydrolysis, lipases, in organic media, are also involved in transformation reactions such as esterification, interesterification, transesterification, alcoholysis, aminolysis and acidolysis [4]. Lipase reactions are versatile, with the enzyme having unique properties of selectivity and substrate specificity. They have the efficiency to catalyze reactions in both aqueous and non-aqueous media as a result of their high stability against a wide range of temperature, pH, and even organic solvents [5, 6].

Lipases are a highly diverse group of enzymes. They are one of the widely used biocatalysts and account for nearly 10 % of the enzyme market [7]. Lipases are used in several processes such as organic chemical synthesis, hydrolysis of fats and oils, flavor enhancement, resolution of racemic mixtures, and chemical analysis, in several industrial sectors such as detergent, food processing, leather, pharmaceutical, wastewater treatment, and biofuel industries [8 – 10].

Lipases are produced by animals, plants, and microorganisms. However, the microbes are preferred as commercial sources of the enzyme due to their reliability, stability, ease of cultivation and manipulation to obtain higher yield [6, 11]. Among microbial sources of lipases, fungi have been recognized as good producers of extracellular lipases, with the enzyme being thermally stable, and processes such as enzyme extraction and purification being relatively easy compared to other sources [9, 12, 13]. Fungal lipases have broad applications in several industries such as leather, textile, cosmetics, biodiesel production, detergent manufacturing, pharmaceutical, pulp and paper, dairy, beverages, medical and diagnostics, and the oleochemical industry [14, 15].

Several genera of fungi, isolated from diverse environments, such as *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium*, *Geotrichum*, *Trichoderma*, and *Mucor* species, have been implicated in lipase production with the conditions for the enzyme production optimized [16 – 20].

The knowledge of new microorganisms, capable of producing lipase, along with the knowledge of their operational conditions will be very helpful in achieving the best combinations for high-value lipase production, and hence lower production costs [21]. Therefore, there is a sustained search for new microbial strains capable of producing copious amount of the enzyme. Enzyme biosynthesis by filamentous fungi is reported to be influenced by numerous factors such as pH, temperature, carbon and nitrogen sources [22, 23]. Therefore, the optimization of media composition, as well as the physical fermentation conditions is essential for enhancing the yield of the enzyme.

As cocoa contains a substantial amount of fats and other nutrients, the probability of having lipase-producing fungi as natural inhabitants in cocoa processing effluent is high. To our knowledge, this is the first study involving the exploration of the effluent for lipolytic fungi. Therefore, the objective of this study is to determine the influence of various physical and nutritional factors on lipase production from the fungus *Mucor* sp. B6 isolated from a Cocoa Processing Plant effluent.

MATERIALS AND METHODS

Isolation and maintenance of culture

Five samples were collected from the different sites of the effluent from a Cocoa Processing Plant located in Ile Oluji, Ondo State, Nigeria. They were immediately transported to the laboratory in a sterile glass bottle. The effluent samples were serially diluted to 10^{-6} , and 1.0 mL of each dilution was plated on the potato dextrose agar (PDA) in triplicates. The Petri plates were incubated at 30 °C for 5 days. Morphologically distinct fungal growths were successively subcultured on fresh medium until pure colonies were obtained. The pure colonies were maintained on PDA slants and stored at 4 °C.

Screening of fungi for lipase production

The pure fungal isolates were cultured on tributyrin agar incubated at 30 °C for 5 days and then screened for triglyceride hydrolysis according to the method described by Wadia and Jain [17]. The clear hydrolytic zones formed around fungal growth were observed and the diameter measured using a meter rule. The fungal isolate with the most appreciable lipolytic activity was selected for further study.

Characterization and identification of lipase-producing fungi

The lipase-producing fungi were identified phenotypically by determining their cultural, morphological and microscopical characteristics. The fungi were characterized macroscopically and microscopically with reference to the Pictorial Atlas of Soil and Seed Fungi [24]. The fungal cultural morphology was studied by observing the features on Petri plate cultures such as surface color, margin, form, texture and diameter. Microscopic characterization was carried out by using a compound binocular microscope. The lactophenol cotton blue-stained slides mounted with a small portion of each of the fungal mycelia were examined. The spore type and shape, type of sporangia and type of hyphae were observed and recorded [25].

Growth and enzyme production

The enzyme production was carried out in a 250 mL Erlenmeyer flask containing 50 mL medium made up of 0.3 % peptone, 0.1 % yeast extract, 0.05 % NaCl, 0.05 % $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 % gum acacia and 1 % olive oil. The pH of the medium was adjusted to 6.0 and the medium was sterilized at 121 °C and $1.05 \text{ kg} \cdot \text{cm}^{-3}$ pressure for 15 min. The fungal biomass and enzyme production of the fungus was determined by inoculating 50 mL enzyme production medium with 0.5 mL standardized inoculum of spore suspension ($5 \times 10^5 \text{ spore} \cdot \text{mL}^{-1}$). These were incubated at 30 °C for 12 days, with agitation at 100 rpm. At 24 h intervals, the culture broth was filtered on a filter paper (Whatman paper No 1) followed by centrifugation at 10,000 rpm at 4 °C for 20 min and the fungal biomass was determined everyday of incubation (24 h interval) according to its dry weight. The supernatant was collected and used as the crude enzyme extract.

Lipase assay

Lipase activity was determined spectrophotometrically using para-nitrophenol laurate (pNPL) as substrate according to Winkler and Stuckmann [26]. The reaction mixture consisted of 700 μL pNPL solution and 300 μL crude lipase extract. The pNPL solution was prepared by adding solution A (0.001 g pNPL in 1.0 mL isopropanol) 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). The progress of the reaction was monitored by the change in absorbance at 410 nm over a 3 min period at 30 °C using a spectrophotometer. Change in absorbance was used in calculating the enzyme activity. One unit of lipase activity (IU) was defined as the amount of enzyme that liberated 1.0 μmol equivalent of pNP per mL per min (ϵ : 15000 $\text{cm}^2 \cdot \text{mol}^{-1}$) under the standard assay conditions. Protein concentration was determined by the method of Bradford [27] using bovine serum albumin as standard.

Influence of physical and nutritional parameters on lipase production

To optimize lipase production from the fungus, the influence of different nutritional and physical parameters on enzyme production was studied:

Effect of carbon sources on lipase production

Various carbon sources (olive oil, palm oil, groundnut oil, coconut oil, palm kernel oil) were supplemented into the production medium, at a concentration of 1.0 % w/v. The medium was then inoculated with a standardized inoculum of the fungal isolate and incubated at 30 °C for 7 days with steady agitation at 100 rpm. After incubation, the cell-free supernatant obtained was assayed for enzyme activity to determine the most carbon source suitable for lipase production [28].

Effect of nitrogen sources on lipase production

Different nitrogen sources such as ammonium sulphate, sodium nitrate, potassium nitrate, peptone and yeast extract, at a concentration of 1.0 % w/v, were supplemented into the production medium. All the media were adjusted to pH 6.0 and sterilized at 121 °C and 1.05 $\text{kg} \cdot \text{cm}^{-3}$ for 15 min. The media were then inoculated with the standardized inoculum of the lipolytic fungal strain and incubated at 30 °C for 7 days with steady agitation at 100 rpm [28].

Determination of optimum pH for lipase production

The optimum pH for the production of lipase was determined by varying the pH of the basal medium from 4.0 to 8.0. The pH of the production medium was adjusted to the different pH condition using 0.1 N HCl or 0.1 N NaOH solution. The medium was sterilized at 121 °C for 15 min at a pressure of 1.05 $\text{kg} \cdot \text{cm}^{-3}$ after which it was inoculated with the standardized fungal inoculum and incubated at 30 °C for 7 days with agitation at 100 rpm in an incubator shaker [28].

Effect of temperature on lipase production

The optimum temperature was determined by culturing the isolate at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, and 55 °C, for 7 days with agitation at 100 rpm in an incubator shaker [27]. The cell-free supernatants obtained were assayed for lipase activity.

Statistical analysis

Data obtained from triplicate experimental determinations were subjected to statistical analysis and expressed as mean $\pm$ standard deviation (SD) using SPSS version 16.

RESULTS AND DISCUSSION

Fungal isolation, characterization, and identification

Eight morphologically distinct fungal strains were isolated from the effluent from the Cocoa Processing Plant located in Ile Oluji, Ondo State, Nigeria. The isolates were successively subcultured on fresh PDA plates to obtain pure cultures. They were then maintained in PDA slants at 4 °C.

Characterization, identification, and screening of lipase-producing fungi

The eight fungal isolates were screened for their lipolytic activity using tributyrin agar (Table 1). Six of the isolates that showed lipolytic activity on tributyrin agar plates were characterized phenotypically by determining the morphological and microscopic characteristics. They were presumptively identified as strains of *Schizosaccharomyces* sp., *Aspergillus* spp., *Fusarium* sp., *Kluyveromyces* sp. and *Mucor* sp. B6. (Table 2). The six lipase-producing fungal strains were then subjected to further screening for enzyme production in liquid culture media, under submerged fermentation conditions, with olive oil as the only source of carbon. The strain *Mucor* sp. B6 exhibited the most appreciable lipase production with lipase activity $22.0 \text{ U} \cdot \text{mL}^{-1}$ (Figure 1). Therefore, it was selected for further study (Figure 1). Several *Mucor* species have been implicated in lipase production such as *Mucor griseocyanus* [29], *Mucor racemosus* [30] and *Mucor* sp. [13].

Table 1. Screening for lipolytic fungal isolates using trybutyrin agar plates

Isolate code	Diameter of zone of hydrolysis [cm]
B1	2.37 ± 0.51
B2	4.36 ± 0.51
B3	3.40 ± 0.36
B4	3.23 ± 0.60
B5	2.73 ± 0.31
B6	4.77 ± 0.51
B7	0
B8	0

Table 2. Characterization and identification of lipase-producing fungi

Isolate code	Characterization		Presumptive identity
	Cultural/Morphological	Microscopic	
B1	White-colored, dense mycelia growth	Heterogenous, conjugated, enclosed budding cells, thin-walled around the cells.	<i>Schizosaccaromyces</i> sp.
B2	Colonies are fluffy, black colored on the surface and reverse side of agar plate	Conidiophores stipes arose from submerged hyphae, up to 50 to 70 μm long, smooth walled but slightly roughened below the vesicle.	<i>Aspergillus</i> sp.
B3	Dense, fluffy, pale white colored growth on plate	Conidiophores short, wine colored, with apical beak, boat-like structures. Conidia segmented.	<i>Fusarium</i> sp.
B4	A carpet-grass growth on agar plates, with a greenish-black color	Conidiophore upright and slender, black, globose, long bearing phialides at the apex radiating from the entire surface.	<i>Aspergillus</i> sp.
B5	Cream-colored, smooth, yeast-like colonies	Formation of budding cells and endospores, elongated	<i>Kluyveromyces</i> sp.
B6	Colonies are fluffy, greyish brown on agar plate	Erect sporangiophores, globose sporangia with well-developed columella. Sporangiospores are hyaline and smooth walled	<i>Mucor</i> sp. B6

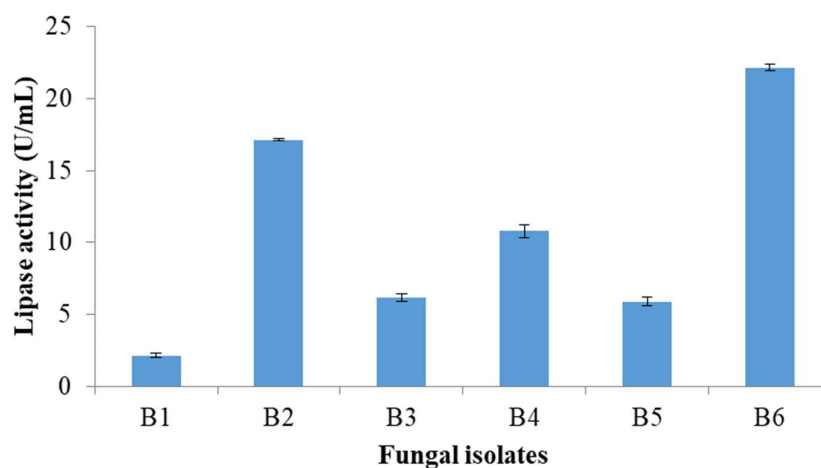


Figure 1. Screening of fungal isolates from effluent from Cocoa Processing Plant for their lipolytic activities

Growth and Lipase Production

Extracellular lipase production from the selected fungus *Mucor* sp. B6 was observed in the culture supernatant of the basal medium after 48 h of incubation with $4.47 \text{ U} \cdot \text{mL}^{-1}$ lipase activity. The enzyme production increased to the maximum after 168 h of incubation with $24.4 \text{ U} \cdot \text{mL}^{-1}$ lipase activity as seen in Figure 2.

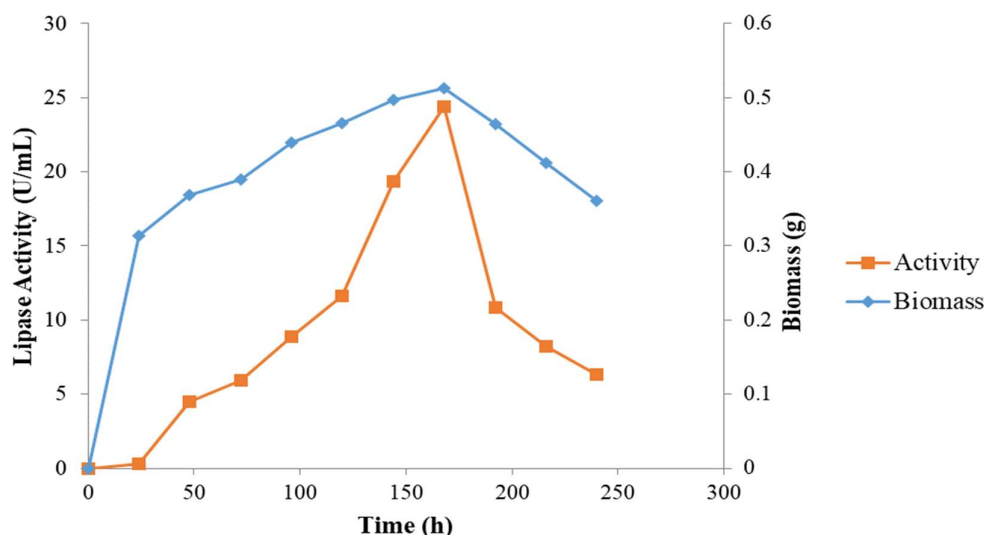


Figure 2. Growth and lipase production curve of *Mucor* sp. B6 at 30°C and pH 6.0

Enzyme productions by most fungi are growth associated and are usually induced in the presence of its substrate in the culture medium. Generally, enzyme production takes place during the logarithmic phase of microbial growth whilst the enzyme levels decrease towards the end of the phase or during the stationary phase. Lipase production by *Mucor* sp. B6 was observed to begin during the log phase in the presence of olive oil as the substrate reached its maximum level after 168 h of incubation period ($24.4 \text{ U} \cdot \text{mL}^{-1}$). The cell population also reached the stationary phase, suggesting that enzyme production was growth-associated. Extracellular lipase production increased with an increase in biomass up to 168 h of incubation near stationary phase after which there was a decline. A decrease in lipase activity was observed during the late stationary phase probably because of a decrease in nutrient availability, and denaturation of the enzyme in the culture medium [31]. Roy *et al.* [32] reported that the maximum lipase production occurred after an incubation period of 214.71 h. However, Ayinla *et al.* [16] reported that an incubation period of 96 h is the optimum for lipase production from *Rhizopus oryzae* ZAC3.

Effect of carbon sources on lipase production from *Mucor* sp. B6

The culture composition such as carbon and nitrogen sources has a significant influence on enzyme production from microorganisms [33]. Microbial lipases mostly are inducible and upon induction secrete extracellular enzymes into the surrounding environment [16].

They are generally produced in the presence of a lipid source such as oil or other inducers such as triacylglycerols, fatty acids, hydrolysable ester, tweens, bile salts and glycerol [34, 35]. Also, the lipid fatty-acid esters are observed to be better inducers of enzyme production relative to the non-lipid substrates such as glucose. Different carbon sources namely palm oil, olive oil, coconut oil, groundnut oil, and palm kernel oil, were used in the production medium at 1 % in this study. Maximum lipase production from *Mucor* sp. B6 was obtained with the use of olive oil as the substrate with lipase activity $18.57 \text{ U} \cdot \text{mL}^{-1}$ (Figure 3). A similar result was reported for the synthesis of lipase from *Fusarium oxysporium* by Hala *et al.* [36], and *Aspergillus niger* by Putri *et al.* [37] where olive oil was found to be the best carbon source. Also, Nunes *et al.* [38], Papanikolaou *et al.* [39], and Ayinla *et al.* [16] all reported maximum lipase production from several filamentous fungal strains when olive oil was used as the substrate.

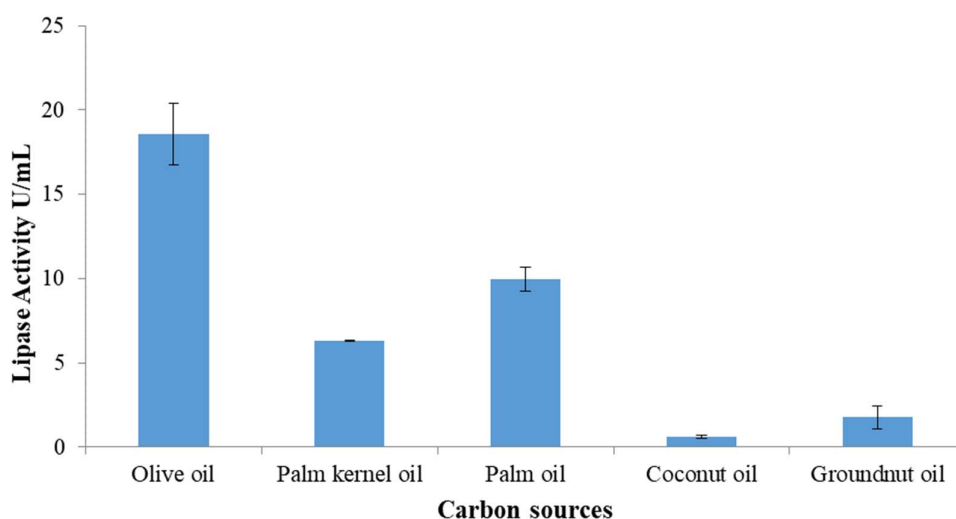


Figure 3. Effect of carbon sources on lipase production from *Mucor* sp. B6 at 30 °C and pH 6.0 after 168 h fermentation period

Effect of nitrogen sources on lipase production from *Mucor* sp. B6

Nitrogen sources play an important role in the biosynthesis of lipase by microorganisms [40]. Yeast extract was observed to be the best nitrogenous source for lipase production from *Mucor* sp. B6 followed by peptone (Figure 4). Most microorganisms utilize both organic and inorganic nitrogen sources for the synthesis of macromolecules such as amino acids, nucleic acids, proteins, and cell wall components [41]. However, the organic sources were observed to result in better enzyme production relative to the inorganic nitrogen sources. Ayinla *et al.* [16] also reported yeast extract as the best nitrogen source for lipase production from *Rhizopus oryzae* ZAC3. Ammonium sulphate produced the highest lipase production among the inorganic nitrogen sources with enzyme activity $2.04 \text{ U} \cdot \text{mL}^{-1}$ (Figure 4).

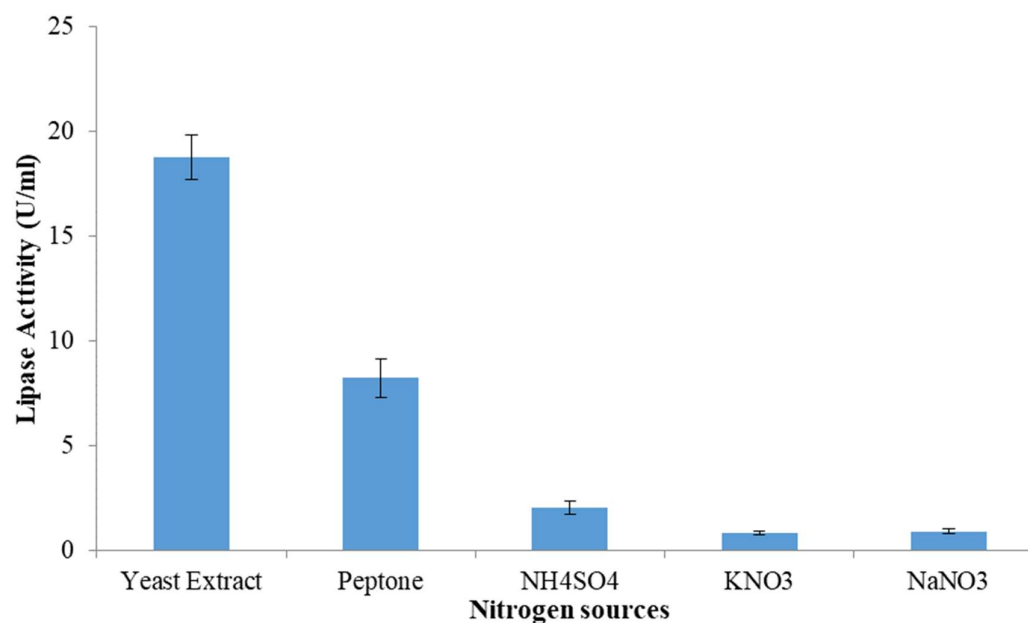


Figure 4. Effect of nitrogen sources on lipase production from *Mucor* sp. B6 at 30 °C and pH 6.0 after 168 h fermentation period

Effect of pH on lipase production from *Mucor* sp. B6

The pH of the production media plays a critical role in the optimal physiological performance of the fungus and the transport of various nutrient components for maximizing enzyme yields. The fermentation medium was subjected to various pH conditions ranging from 4.0 to 9.0. The optimum pH for lipase production was observed to be 6.0 (Figure 5).

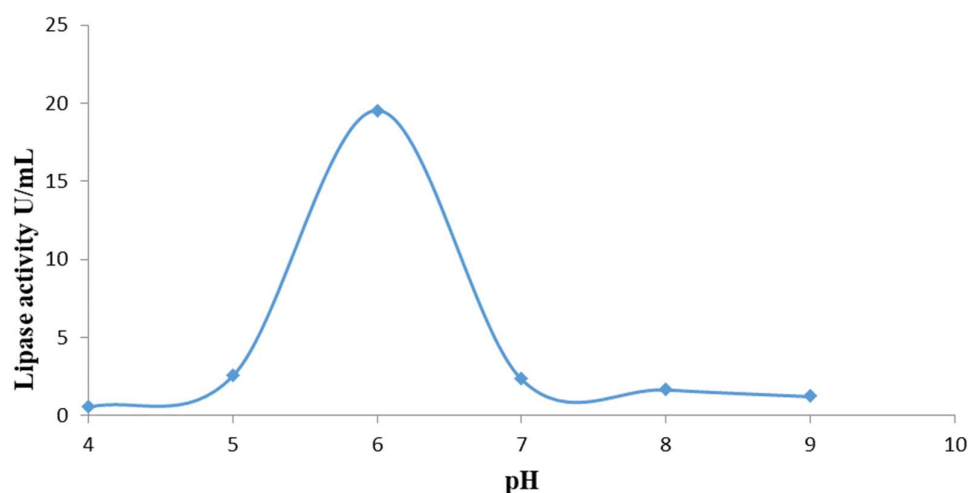


Figure 5. Effect of pH on lipase production from *Mucor* sp. B6 at 30 °C after 168 h fermentation period

Beyond this *pH* level, lipase production declined. This implies that at neutral or alkaline *pH*, growth of, and lipase production from, *Mucor* sp. B6 were negligible. Optimum lipase production at *pH* 6.0 was reported for *A. niger* [42] while *pH* 7.0 was observed to be the best for lipase production from *A. acculeatus* Ms.11 [43].

Effect of temperature on lipase production from *Mucor* sp. B6

Temperature is one of the important environmental factors which influence lipase production. The maximum level of lipase production from the fungus was at 30 °C (Figure 6). Also, Triswayati and Ilmi [43], and Moataza *et al.* [44] reported the optimum temperature for lipase production from the strains *Aspergillus aculeatus* Ms.11, and *Fusarium oxysporium*, respectively, to be observed at 30 °C. However, the optimum temperature 32 °C was observed for lipase production from fungal isolates from oily residues [45] while Roy *et al.* [32] reported the optimum temperature for lipase production from a strain of *A. acculeatus* to be 35 °C. The variation in optimal temperature for enzyme production could be due to the difference of producing microorganisms, the individual microbial metabolism, and their natural habitat [45].

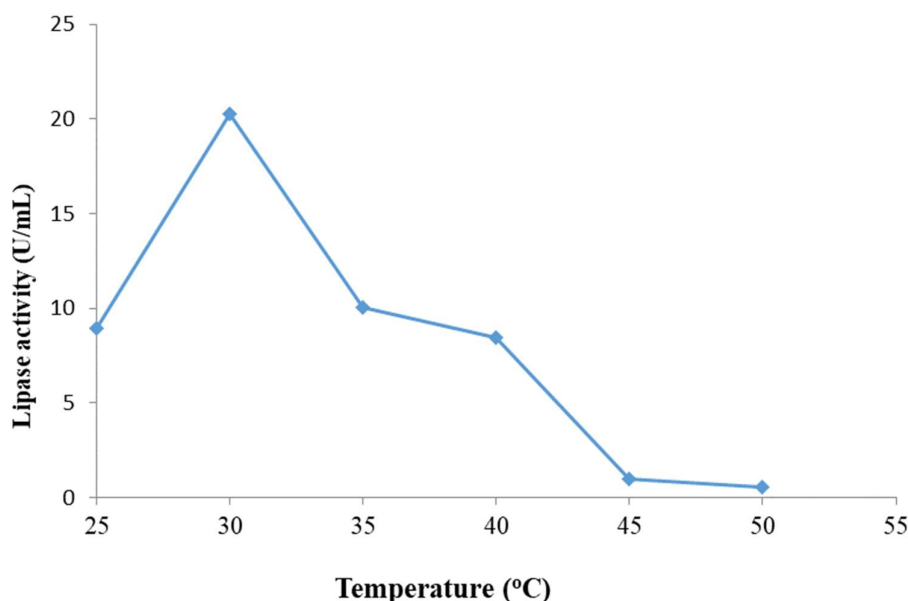


Figure 6. Effect of temperature on lipase production from *Mucor* sp. B6 at *pH* 6.0 after 168 h fermentation period

CONCLUSIONS

From the present study, it could be seen that the fungal strain *Mucor* sp. B6, isolated from cocoa processing plant effluent, has great potential for large-scale production of the industrially-important lipase. The fungus produced maximum lipase with the use of olive oil as an inducer carbon source, and yeast extract as a nitrogenous source. Optimum parameters for the maximum production of the enzyme from the fungus were the 168 h fermentation period, and the *pH* and temperature conditions 6.0 and 30 °C, respectively.

Further studies on the purification and biochemical properties of the enzyme will reveal its suitability for biotechnological and industrial applications.

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