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Production, Partial Purification, and Characterization of Lipase from *Aspergillus niger* and Its Application in Treatment of Vegetable Oil Effluent

Chimaobi Sunday Vincent[1, *], Charles Ogugua Nwuche[2], Mida Habila Mayel[3], Sabinus Oscar Onyebuchi Eze[1]

[1]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Enugu, Nigeria

[2]Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Enugu, Nigeria

[3]Department of Biochemistry, Federal University, Wukari, Nigeria

Email address:

vinchima22@yahoo.com (C. S. Vincent), charles.nwuche@unn.edu.ng (C. O. Nwuche), mayel.mida@fuwukari.edu.ng (M. H. Mayel), sabinus.eze@unn.edu.ng (S. O. O. Eze)

[*]Corresponding author

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Abstract: Vegetable oil factories generate considerable quantity of waste. The wastewater from the production process causes severe environmental pollution. Although, several conventional and biological treatment methods have been tested, the outcomes are usually fraught with significant draw backs. The enzymatic method was employed in the present study due to their many beneficial attributes. Lipase was produced by submerged fermentation using the effluent as a sole source of carbon. Upon partial purification, a 3-fold increase in lipase activity was obtained. The enzyme reacted optimally at 50°C, pH of 6.0 and substrate concentration of 7mg/ml. The crude ezyme displayed identical characteristics with the partially purified one. The crude and partially purified lipase were used to treat the wastwater separately. From the results, crude lipase liberated higher fatty acids (1.0591%) from the vegetable oil effluent than the partially purified lipase (0.6066%) did. Kinetic parameters of the enzyme indicate a K_m value of 8 mg/ml and V_{max} value of 344.83 µmol/min. The results of this study could be applied for the purpose of formulating appropriate policy directives that will aid clean-up of pollution from vegetable oil factories. In addition, vegetable wastewaters can be used as substrates for the production of lipases which could be processed for industiral and biotechnological applications.

Keywords: Lipase, Vegetable Oil Effluent, *Aspergillus niger*, Submerged Fermentation

1. Introduction

Edible refined oil processing industry is a major issue of environmental concern in developing countries [32]. Refining crude vegetable oils generates large amounts of wastewater [39]. The wastewater come mainly from the degumming, deacidification, deodorization and neutralization phases of processing. Pretreatment of oilseeds is a major step in refining and modification of oils. Vegetable oil facilities require significant amounts of water for crude oil production, cooling of the plant, chemical neutralization, washing and deodorization. These operating conditions determine the amount and characteristics of the products and wastes formed [40]. Wastes from vegetable oil refining factories contain oils, fats, soap, polluted effluents and sludges which cause deterioration of the environment and human health [42]. Soap stock can be solid or liquid [14] and is an emulsified aqueous mixture of glycerides and FFAs [30]. The emulsion can contain about 50% of water [41]. No matter how effective the method of processing, vegetable oil effluent

discharged from a factory is objectionable and could pollute streams, rivers or surrounding land [28]. When discharged into water bodies, it makes the water smelly, slimy and inhibits sunlight and oxygen penetration into the water phase [4], resulting in the mortality and morbidity of fishes and other aquatic organisms [11]. In addition, the development of mutagenic and carcinogenic conditions in plants, animals, and man have been reported [16].

Oil and grease reduce the biological activity of treatment processes because the oil film forms around the microbes and on water. This condition leads to decrease in dissolved oxygen levels in the water. Wastewaters from vegetable oils contain significant amounts of fatty acids, oil and grease which are reported to be challenging to treat [17]. The presence of oil and grease in water treatment units cause fouling in process equipment, complication in water discharge requirement and problems in the biological treatment stage [2]. Several approaches have been employed in wastewater treatment to restore impacted environment [12]. The conventional physical and chemical methods suffer from recognisable draw backs [23] while the biological options require the involvement of live autochthonous or specially formulated micro-organisms (allochthonous) to degrade all or certain components of the effluent [29]. Many times, the physical conditions of the polluted environment limit the capability of proven microbial species to perform, there by rendering the whole clean-up operation ineffective and expensive to run and maintain. Biochemical methods (enzymes) have recently gained more attention due to their ease of application and capacity to overcome the stated limitations [18]. The wastes from lipid-processing factories and restaurants can be cleaned by the help of lipases from different origins. Lipase producing strains play a key role in the enzymological remediation of polluted soils [19]. Although lipase can be sourced from plant, animal and microbes, microbial lipase occupies a place of prominence among other biocatalysts owing to their ability to catalyze a wide variety of reactions in aqueous and non-aqueous media [9]. Lipases produced by fungi are typically extracellular and therefore relatively easy to recover after the fermentation [37]. Many genera have been noted as producers of lipase with desirable properties. Today, they are used in a variety of industrial and biotechnological applications. Fungal lipases are derived mainly from *Aspergillus sp*. [33]. The present study examines the production, partial purification and characterization of lipase from a strain of *Aspergillus niger* and its application in the treatment of vegetable oil effluent.

2. Material and Methods

2.1. Microorganism and Culture Conditions

Lipase producing strain of *Aspergillus niger* previously isolated from vegetable oil contaminated soils was obtained from stock collection and resuscitated on potato dextrose (PDA) media for 5 to 7 days. Spores from the fungus were subcultured repeatedly on the same medium to reactivate the cultures. Fresh, young, actively growing spores of the mould were harvested and used for the experiments.

2.2. Prpduction and Extraction of Lipase by Submerged Fermentation (SmF)

Lipase was produced by submerged fermentation. The components of the production media were maintained as specified by Adham and Ahmed [1]. Vegetable oil mill effluent was used as inducer. Using sterile inoculation loop [26], 5 days old cultures of the fungi actively growing on PDA slants were harvested in 10 ml of sterile distilled water. The black-coloured spore suspension was adjusted to a spore concentration of 1×10^{14} spores/ml using a Neubauer haemacytometer. The standardized spore inoculum (5% v/v) was aseptically inoculated into 250 ml Erlenmeyer flasks containing 50 ml of the production medium and incubated at 29°C under continuous shaking (MK-V orbital Electronic Shaker) for 72h at 200 rpm. The crude enzyme was separated from the fermentation broth by passing through muslin cloth under ice [34], followed by centrifugation at 4000 rpm (Model 800 Centrifuge Machine) for 15 min. The pooled supernatant was stored at 4°C for further use as the crude enzyme.

2.3. Enzyme Assay

Assay was done following the colorimetric method of Duncombe [7]. One unit of lipase activity was defined as the number of micromoles of fatty acids released per minute under the assay conditions.

2.4. Specific Activity

Specific activity was calculated by dividing enzyme activity (U/mL/min) with total protein produced (U/mg).

2.5. Protein Content Determination

Protein content was determined by the method of Lowry *et al*. [21].

2.6. Ammonium Sulphate $(NH_4)_2SO_4$ Precipitation

The crude lipase was subjected to 70% ammonium sulphate saturation by slowly adding solid ammonium sulphate to the solution of crude enzyme in a 1L beaker over a period of 1h. The solution was gently stirred (JB-3 Magnetic Stirrer) and kept standing afterwards at 4°C for 36 h. The precipitates were collected after centrifugation at 5000 x g for 20 min. Enzyme activity was determined.

2.7. Dialysis

The dialysis was carried out in a dialysis bag according to Dixon and Webb [6]. The precipitates obtained after centrifugation were dissolved in minimal buffer making up a total of 30ml solution. The solution was dialysed for 24h in 0.15M phosphate buffer (pH 5.9) inside a beaker placed on a magnetic stirrer (JB-3) to ensure thorough mixing. The buffer was changed every 6 h.

2.8. Application of Lipase in the Treatment of Vegetable Oil Effluent

One milliliter of the crude enzyme preparation was pipetted into various volumes of the effluent (10, 20, 30, 40 and 50ml) in 250ml Erlenmeyer flask in triplicate experiments. Also, 1ml of the partially purified enzyme was pipetted into separate volumes of the effluent as already described. The control contained 1ml of distilled water in the various volumes of the effluent (10, 20, 30, 40 and 50ml) in 250ml Erlenmeyer. The mixtures were incubated overnight on MK-V orbital shaker (200rpm) at 28°C.

Determination of Free Fatty Acid Released: After incubation of the reaction mixture, the released free fatty acids were measured following the protocol of AOAC [3].

2.9. Studies on Partially Purified Enzyme

2.9.1. Effect of Temperature on Lipase Activity

The temperature dependence of lipase activity was measured by incubating the 0.5 ml of the enzyme in SS-H water-bath at varied temperatures (20, 30, 40, 50, 60, and 70°C). The activity was determined after 30 min intervals.

2.9.2. Effect of pH on Lipase Activity

The activity of lipase was determined under different pH range (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) with sodium phosphate buffer (0.15M) and olive oil as substrate. The effect of pH on lipase activity was determined by incubating 0.5 ml of the lipase in the different buffer solutions for 30min at 37°C in SS-H water-bath. The activity was then determined.

2.9.3. Effect of Substrate Concentration on Lipase Activity

The effect of substrate concentration on the activity of the enzymes was determined by varying the concentration (1, 3, 5, 7, 9 and 11mg/ml) of the substrate during preparation of the substrate emulsion. The activity of the enzyme was determined.

3. Results and Discussion

3.1. Morphological and Microscopic Identification of the Isolated Mould

Vegetable oil effluent dumpsites constitute one of the many areas where lipolytic microbes thrive. The isolated fungus was enriched by periodic sub-culturing on PDA. Identification was on the basis of morphological properties (e.g., colony colours, reverse side and margins) which is one of the oldest and most adopted tools [8]. The culture displayed an umbonate and entire elevation and margin respectively (Figure 2). It had rapid growth pattern, a colourless reverse side (Figure 3) and dark brown to black surface colouration (Figure 2) suggesting the species could be *Aspergillus niger* [8]. Under microscopic examination, the fungi showed the conidia heads to be darkish-brown and the ornamentation exine spiny. These features are typical of *Aspergillus niger.*

3.2. Screening for Lipolytic Activity

When the species was screened with Tween 80, white zone appeared around the colony (Not shown). The visible precipitate is due to the deposition of calcium salt crystals formed by the liberated fatty acids due to the action of lipase. Subash *et al*., [38] suggested that the primary role of calcium ions is to remove fatty acids formed during hydrolysis as insoluble calcium soaps thus changing the substrate-water interface relationship to favorable conditions for enzyme action.

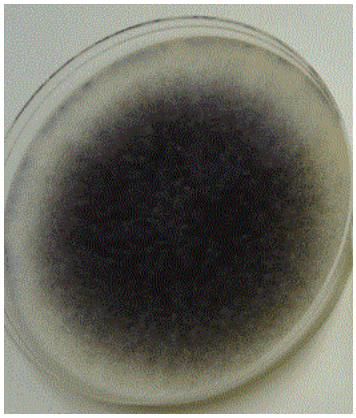

Figure 1. *Microbial growth after 72 h.*

Figure 2. *Rapid growth of the microbe.*

Figure 3. *Back side of the petri-dish after 72 h.*

3.3. Purification

Maximum lipase recovery is usually achieved at 60 to 70% ammonium sulphate saturation [5]. Purification of the *A. niger* lipase at 70% ammonium sulphate (Figure 1) followed

by dialysis resulted in a 3.0-fold increase in lipase activity with specific activity of 373.8717Umg^{-1} (Table 1). Results of lipase activity obtained in the present study agree with Lívia *et al*., [21] who had 2.91-fold increase after double purification, although the specific activity (3.5×10^4 Umg^{-1}) reported was far higher than data obtained (373.8717Umg^{-1}) from our studies. Other authors have reported different activity values for their purified lipases. For Safaradeen *et al.,* [31], an increase in specific activity from 5.29 to 20.8 Umg^{-1} was achieved with a purified lipase after a two-step purification protocol that resulted in 3.93-fold increase in activity. The partially purified lipase studied by Shafei and Allam [36] displayed a 4.10-fold activity over the corresponding crude extract.

Table 1. *Purification Table.*

Purification step	Vol. (ml) of enzyme	Protein Conc.(mg/ml)	Activity (U/ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification fold
Crude Enzyme	500	3.0743	266.3158	1537.15	133157.9	86.6265	1
70% $(NH_4)_2SO_4$ ppt	30	1.4617	175.4386	43.851	5263.158	120.0238	1.39
Dialyzed Enzyme	40	0.6241	233.333	24.964	9333.332	373.8717	3

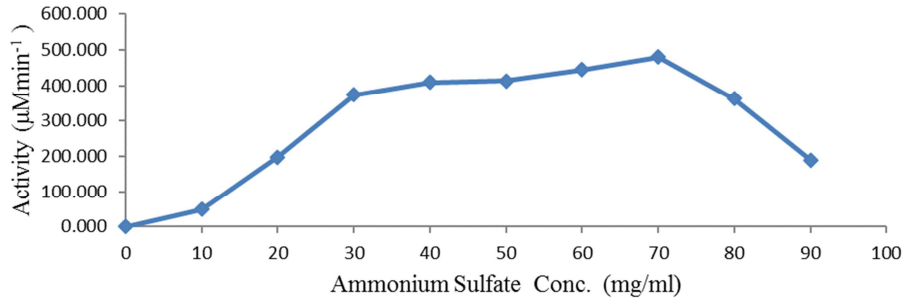

Figure 4. *Ammonium sulphate profiling.*

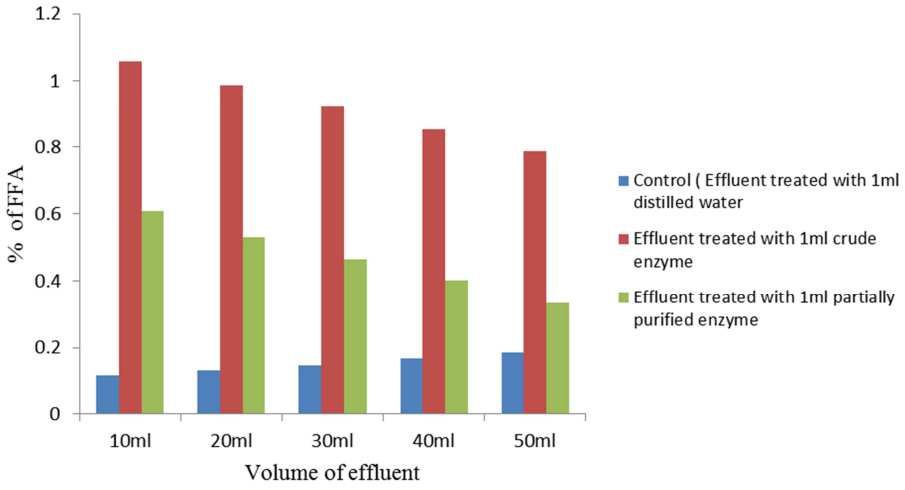

Figure 5. *Concentration of FFA released after treating the effluent with the crude and partially purified lipase.*

3.4. Effluent

The use of lipases in industries is enormous and increasing [27]. Natural oils stimulate lipase production. Elevated level of lipase production by *Bacillus sp*. was observed upon using olive oil as substrate in the culture medium. Vegetable oil effluent is rich in oil and grease. These components are lipase inducers. The high percentage of FFA released by crude lipase over the partially purified lipase probably come from the higher loading capacity since the crude extract contained higher protein and higher lipase concentration. Oil and grease are potential inducers. The low % of FFA in the partially purified lipase indicate a stream-lining of the inducers during purification. This result is in harmony with other findings. Jun *et al*., [13] applied crude lipase of *P. aeruginosa* SL-72 to wastewater contaminated with crude oil, resulting in degradation of 82.83% of the oil content and 86.39% reduction of COD after 7 days of treatment.

3.5. Characterization

Lipase from *Aspergillus niger* are optimally active within a temperature range of 40 to 60°C [10]. In the present study, optimal activity was recorded at 50°C (Figure 6). The activity of the lipase decreased rapidly at temperatures above 60°C and was totally lost at 90°C. Overall, lipases from *A. niger* strains have been reported to be active between 40 and 55°C [24]. The study

demonstrates that the lipase is weakly acidic (Figure 7). This result is consistent with earlier report by Nehad and Ahmed [25] who obtained optimum lipase activity of the enzyme extract at pH 6. According to their finding, when lipase activity was measured at a pH range from 5.7 to 9.0 using phosphate and Tris-buffer, the enzyme was quite active over a pH of 6.0 – 7.5. These results are similar to other reported lipases which had optimum pH between 6.5 and 8.5 [35].

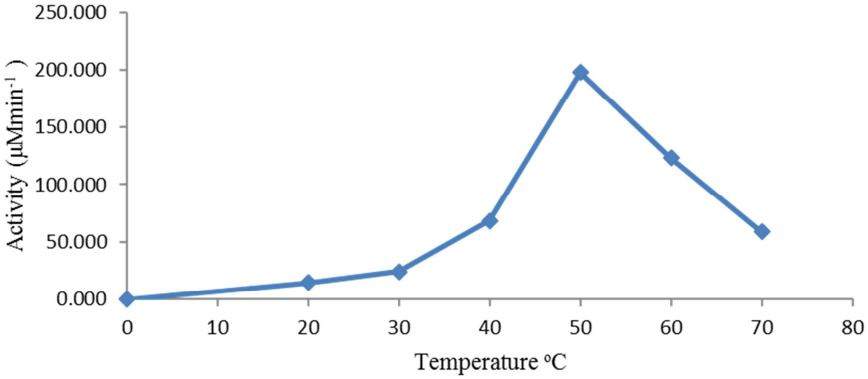

Figure 6. *Effect of temperature on lipase activity.*

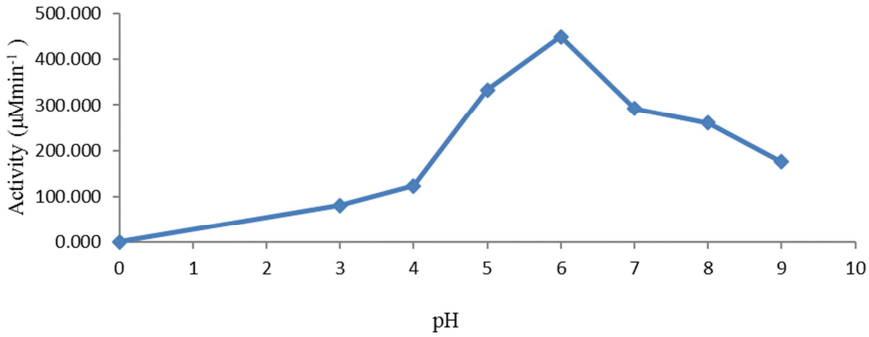

Figure 7. *Effect of pH on lipase activity.*

Result of the study on the effect of substrate concentration on the *A. niger* lipase demonstrated that there was a direct relationship between lipase activity and substrate concentration (Figure 8) up to a saturation point of 7mg/ml of the substrate. The decline after this concentration may be due to enzyme-substrate ratio, enzyme inhibition by substrate or changes in physicochemical characteristics of enzyme or substrate [15]. The result obtained in this study could be very useful in the manipulation of industrial bio-catalytic reactions involving lipases from *A. niger* and other microbes.

3.6. Kinetic Parameters

The kinetic constants for the lipase were calculated from double reciprocal plot. The K_m and V_{max} of 8 mg/ml and 344.83 µmol/min. were obtained for the lipase. Mayel *et al*., [22] reported Km and Vmax of 17.54 mg/ml and 769.23 µmole/min. respectively for lipase from *Aspergillus nidulans* while Km and V_{max} of 9.71 mg/ml and 714.29 µmole/min were reported for lipase from *Aspergillus niger*. The Km of this lipase (Figure 9) suggests the enzyme exhibited high affinity for the substrate.

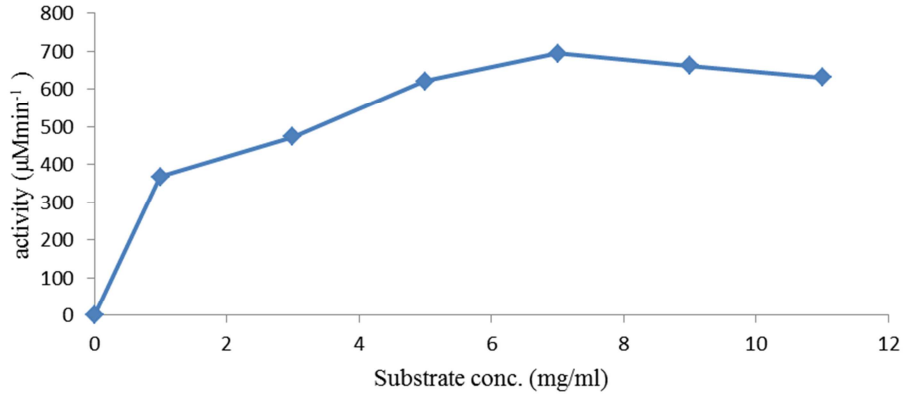

Figure 8. *Effect of substrate conc. on lipase activity.*

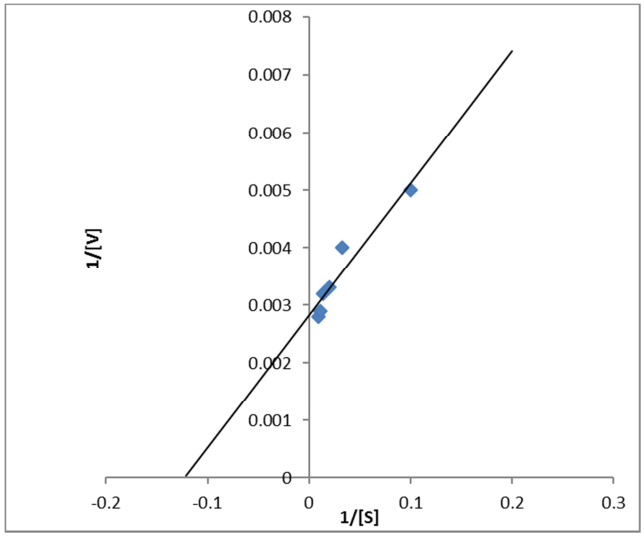

Figure 9. *Lineweaver-Burk Plot for Lipase.*

4. Conclusion

The study reveals that vegetable oil effluent can be valorized as a source of carbon for lipase production from *Aspergillus niger*. The enzyme produced (lipase) can as well be used to treat vegetable oil mill effluent. The result showed that crude enzymes appear more effective in treatment involving lipid rich wastes than the partially purified preparation.

5. Recommendations

The following further studies are recommended to be carried out on the studied isolate and enzyme produced:

i. Optimization of different inducers for lipase production by these very isolates.
ii. Optimization of the type of fermentation applied (SmF or SSF).
iii. Genetically modifying the isolates for better yields.
iv. Purification of the enzymes to the level of gel filtration chromatography, ion exchange chromatography and SDS-gel electrophoresis.
v. Determination of the molecular weight of the enzymes and
vi. Structural elucidation of the active sites.

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