Lipase Production from Palm Oil Mill Effluent Using Altered Fungal Isolate Through Chemical Mutagenesis

Cyprian Erumiseli Oshoma1, *, Elvis Eseosa Osawaru2, Osayi Brenda Isichei-Ukah1, Henry Uzo Oshilonyah3

1Department of Microbiology, University of Benin, Benin City, Nigeria
2Biochemistry Division, Nigerian Institute for Oil Palm Research, Benin City, Nigeria
3Department of Medical Microbiology, Central Hospital Laboratory, Agbor, Nigeria

Email address
cyprian.oshoma@uniben.edu (C. E. Oshoma)
*Corresponding author

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Abstract: Lipases of microbial origin represent the most widely used class of enzymes in biotechnology. This study was aimed at production of lipase by using chemically altered strains of Aspergillus niger isolated from Palm Oil Mill Effluent (POME). The fungus spores were treated with nitrous acid (HNO2) and N-methyl-N’nitro-N-nitroso guanidine (NTG) for strain improvement. The mutated strains were evaluated for lipase production via fermentation of POME. Fungal growth, free fatty acid percentage, lipase activity and pH were analyzed in the course of fermentation using standard procedures. The result of the mutants and their lipase activity showed that Nitrous acid mutant (NA50) had the highest fungal growth and lipase activity to be 16.07 ± 0.03 x 10^6 cfu/mL and 11.69 ± 0.07 U/mL respectively. The lipase yield of the mutant NA50 was 39.00% improvement than the wild strain. Statistically, comparing lipase activity of NA50 strain to others showed a significant difference (p< 0.05). Subsequently, NTG mutants showed that strain NN120 had a 22.65% improvement in lipase activity over Nitrous acid mutant (NA50) strain and a 70.63% improvement over the wild strain A. niger. The results indicated that POME can be utilized for lipase production and the process can be enhanced through strain improvement using nitrous acid and N-methyl-N’nitro-N-nitroso guanidine

Keywords: Lipases, Palm Oil Mill Effluent (POME), Mutagenic Chemicals, Strain, Fermentation

1. Introduction

A major environmental issue of global concern is effective management of agricultural effluent such as Palm-oil mill effluent (POME) and Olive mill effluent [1]. Current observation is that the global intensification of agricultural products has resulted in the production of large quantities of food as well as generating lot of agricultural effluent as waste [2]. Improper management of the effluent can result to public health risk and environmental problems such as air pollution and diseases [3]. Palm-oil mill effluent (POME) is an organic residue generated as waste product during the extraction of palm oil from fresh palm fruits. The effluent is known with high compositions of total solids, suspended organic solids, dissolved organic matter among others [4] that can be utilized as substrate for microorganisms [5]. The utilization of this cheap available substrate to useful products will help in reducing environmental problems caused by this effluent. Thus, the conversion of this cheap readily available substrate by microorganisms into value added products like enzyme will enhance sustainable development [6].

Lipases catalyze the esterification of glycerol from mono, di and triglycerides [7]. These enzymes are widely distributed in nature and have been found in many species of animals, plants, bacteria, yeasts and fungi [8]. They are employed in waste water treatment (degreasing of lipid clogged drains), pharmaceutical (resolution of racemic
mixtures), dairy (hydrolysis of milk, fat), leather (removal of lipids from hides and skin), detergent (removal of oil / fat stains) and medical (diagnostic tool in blood triglyceride assay) industries [9].

Fungi are preferable lipase sources because they are usually excreted extracellularly, facilitating extraction from fermentation media with Aspergillus species being more widely used as sources of lipases [10]. Increase in secretion of these enzymes by the selected strains requires changes in genetic characteristics by spontaneous or induced mutation. These mutated strains reduce the cost of fermentation as the organism increases its productivity as well as possession of specialized desirable characteristics [11, 12].

The increasing demand for lipases to be applied in various biotechnological processes has called for enhancement in both qualitative improvement and quantitative increment in the production of lipases [9, 13]. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme, as the quantities produced by wild strains are low. Strain improvement is an essential part of process development for fermentation products [14]. Developed strains can reduce production costs with increased productivity and can possess some desirable specialized characteristics. Such improved strains can be obtained by mutation and selection depending on the alternate processes of diversification, selection and re-diversification, so that better strains are successfully picked out and further improved [15]. Mutation introduces changes in DNA present in the nucleus of the organism, which can lead to increased productivity [16]. Toscano et al. [17], reported that induced mutagenesis through the use of chemical mutagens is a key step for the development of effective technologies for the industrial scale production of lipases. Therefore, this study was aimed at the production of lipase from POME using altered Aspergillus niger through chemical mutagenesis such as nitrous acid and N-methyl-N′nitro-N-nitroso guanidine.

2. Materials and Methods

2.1. Sample Collection

Palm Oil Mill Effluent (POME) samples, a day old, were collected from the Oil Mill Division of the Nigerian Institute for Oil Palm Research (NIFOR) Benin City, Nigeria. The fresh POME samples were carefully collected into 4.5 L plastic container previously cleansed and rinsed with 70% ethanol and sterile distilled water.

2.2. Inoculum Preparation and Size

The fungus strain A niger used in this study was isolated and selected due to the previous investigation for lipase production among other screened fungal isolates [18]. The strain was maintained on potato dextrose agar (PDA) slant and stored at 4°C. The inoculum was prepared from subcultured A. niger on potato dextrose agar (PDA) plates that were incubated for 5 d. The A. niger cultured plate was flooded with 10 mL of sterile 1% v/v tween 80 solution to dislodge the spores from the hyphae. The solution with spores was filtered with sterile muslin cloth to remove any hyphal fragments present [19]. The number of spores were counted using a haemocytometer and inoculum size of 10^6 spore/mL of each fungal spores was used to inoculate all the media respectively.

2.3. Mutation of Fungal Isolate with Nitrous Acid (HNO2) Treatment

Aspergillus niger was subjected to nitrous acid (HNO2) treatment according to the modified procedure of Karaman and Medicherla [11]. To 9 mL of 10^6 dilution of A. niger spore suspension, 1 mL of sterile stock solution of 0.01 M sodium nitrate was transferred. From the aliquots 1 mL was withdrawn every 10 min intervals till the 50th min. Phosphate buffer (0.5 mL) was added to each sample and 0.1 M sodium hydroxide (NaOH) was used to neutralize the nitrous acid. The exposed suspension (0.1 mL) was plated on potato dextrose agar medium and the suspension was uniformly distributed using a sterile spreader. Each HNO2 exposed spore suspension was stored overnight to avoid photo reactivation. The plates were incubated for 5 days at 28°C. The selected mutant strains on the basis of their morphology, size and shape was further streaked PDA plates incubated for 5 days at 28°C. The treated spores (HNO2) suspension were used for the fermentation processes.

2.4. N-methyl-N’nitro-N-nitroso Guanidine (NTG) Treatment

The mutant strain (HNO2 treated) with the highest lipase producing ability was then selected for N-methyl-N′nitro-N-nitroso guanidine (NTG) treatment. To a 9 mL of spore suspension, 1 mL of NTG (3 mg mL^-1 in phosphate buffer) was added. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 30, 60, 90, 120, 150, 180 min and immediately centrifuged for 10 min at 5000 rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and suspended in 10 mL of sterile phosphate buffer. The samples were serially diluted in the same buffer and 0.1 mL of this NTG treated suspension was plated on potato dextrose agar medium and the suspension was uniformly distributed using a sterile spreader. Each NTG treated spore suspension was stored overnight to avoid photo reactivation. The plates were incubated for 5 days at 28°C. The selected mutant strains on the basis of their morphology, size and shape was further streaked PDA plates incubated for 5 days at 28°C. The treated spores (NTG) suspension were used for the fermentation processes.

2.5. Fermentation Process

Freshly collected POME samples were mixed with distilled water in the ratio 1:2. The mixed samples were passed through muslin cloth to trap solids leaving behind the broth. A 100 mL volume of the broth was transferred into 250 mL Erlenmeyer flasks. The samples thus prepared were
autoclaved at 121°C for 15 min. Samples were prepared in duplicates and were designated palm oil mill effluent broth. The flasks containing the media were inoculated with 500 uL of each wild and mutated strains of *A. niger* respectively with inoculum size of 10^6 spore/mL. The media were left to ferment on an orbital shaker at 120 rpm at temperature of 28±2°C followed by determination of fungal growth, lipase activity, FFA and pH of medium at every 2 days interval for 8 days.

### 2.6. Analytical Methods

Fungal growth was determined using pour-plated method on potato dextrose agar (PDA) in duplicate. Aliquot of 1 mL of the appropriate dilution was pour plated in potato dextrose agar. The plates were incubated at 28±2°C for 72 h. All plates were counted after incubation period and recorded as cfu/mL. Lipase activity was measured by modification of the titrimetric assay method of Pignede *et al.* [20]. The substrate (primary) emulsion was prepared with olive oil, gum arabic and water (4:1:2). Appropriate measure of olive oil and gum arabic was triturated with pestle in a clean dry porcelain mortar. When well mixed, the vehicle (water) was added all at once and triturated vigorously to produce a thick white creamy emulsion; the primary emulsion. The supernatant of the cell culture (20 µL) was added to 5 mL of substrate emulsion and 2 mL of 50 mM phosphate buffer (pH 7.0 (Na_2HPO_4-KH_2PO_4)) before incubating for 20 mins at 37°C with shaking (120 rpm). The reaction was stopped with 4 mL of acetone-ethanol (1:1 v/v) containing 2-3 drops of 0.09% phenolphthalein as indicator. Enzymatic activity was determined by titration of fatty acid released with 50 mM NaOH. The end point was light pink in colour. All lipase activity assays were performed in triplicate. One unit of lipase was defined as the amount of enzyme that catalyzes the release of 1µmol of fatty acids per minute at 37°C. Free fatty acid was determined by dispensing 10 mL of the sample into conical flasks and adding 3 drops of phenolphthalein indicator. Sodium hydroxide (0.1N) was titrated against ten millilitres of POME to pH of 9.5 and the volume of NaOH used was quantified [21]. The Free Fatty Acid was calculated using modified method of Kanimozhi *et al.* [22]. Determination of pH was through the use of pH meter (3305 Jenway, England).

### 2.7. Statistical Analysis

All assays were carried out in duplicates, means and standard deviations (SD) were determined using SPSS version 23. However, t-test was used for statistical comparison of the data for fungal growth and lipase production from the different isolates.

### 3. Results

The fungus (*A. niger*) growth exposed to nitrous acid at different time durations (10, 20, 30, 40 and 50 mins) is shown in Figure 1. Exposure of *A. niger* to nitrous acid for 50 min gave highest value of 16.07 ± 0.03 x 10^6 cfu/mL and the least (12.80 ± 0.14 x 10^6 cfu/mL) was form strain NA10 on day 8. This denotes a 14.29% increase for NA50 than wild strain with the ability to utilize POME for biomass production by the mutant *A. niger*.

![Graph showing fungal growth](image)

**Figure 1.** Effect of nitrous acid on fungal growth produced by *A. niger* during fermentation of POME. Values are means ± standard error of duplicate measurements. NA10 – NA50 are selected strain exposed to nitrous acid at specified time (min) while wild without exposure.
The percentage free fatty acid yield during the fermentation is shown in Figure 2. The highest yield of 32.54 ± 1.56 followed by 27.91 ± 0.04 and the least 21.58 ± 0.21% were observed in the mutant strains of NA50, NA40 and NA10 respectively on day 8. The lipase activities of the various mutants of \textit{A. niger} in POME is shown in Figure 3. It was observed that the lipase activity increased consistently for all the treatments but NA50 had the highest lipase activity of 11.69 ± 0.07 U/mL while the NA10 strain had the least lipase activity (7.65 ±0.04 U/mL). Statistically, comparing lipase activity of NA50 strain to others showed a significant difference (p< 0.05).

![Figure 2](image2.png)

**Figure 2.** Effect of nitrous acid on Free Fatty Acid (FFA) (%) produced by \textit{A. niger} during fermentation of POME. Values are means ± standard error of duplicate measurements. NA10 – NA50 are selected strain exposed to nitrous acid at specified time (min) while wild without exposure.

![Figure 3](image3.png)

**Figure 3.** Effect of nitrous acid on Lipase activity (U/mL) produced by \textit{A. niger} during fermentation of POME. Values are means ± standard error of duplicate measurements. NA10 – NA50 are selected strain exposed to nitrous acid at specified time (min) while wild without exposure.

The nitrous acid \textit{A. niger} mutant NA50 was selected and further subjected to stain improvement by N-methyl-N’nitro-N-nitroso guanidine at various time interval (30, 60, 90, 120, 150 and 180 min). The \textit{A. niger} growth for the treated N-methyl-N’nitro-N-nitroso guanidine strains is shown in figure 4. The mutant NN120 had the highest fungal growth of 21.83 ± 0.06 x 10^6 cfu/mL while the NN30 strain had a least (15.99 ± 0.06 x 10^6 cfu/mL).
The result of % Free Fatty Acid released from POME using the various NNG mutant strains is shown in figure 5. The highest yield (37.76 ± 0.76%) was observed from NN120 strain while the least (30.84 ± 0.54%) was from NN30.

The levels of lipases production were observed to be dependent on *A. niger* exposed to N-methyl-N’nitro-N-nitroso guanidine at different durations (30mins, 60mins, 90mins, 120mins, 150mins and 180mins). The lipase activities of the various nitrous acid and N-methyl-N’nitro-N-nitroso guanidine treated mutants of *A. niger* in POME is shown in figure 6. It was observed that the lipase activity increased consistently for all the treatments however, NN120 had the highest lipase activity of 14.35 ± 0.39 U/mL while the least (11.70 ± 0.08 U/mL) was from NA50 (control) strain. This implies that there was a 20.88% increase in the lipase activity of NN120 in compared to NA50 (control) strain and a 65.53% increase in its lipase activity compared to the wild strain *A. niger*. Hence, further exposure of the nitrous acid treated *A. niger* (NA50) to N-methyl-N’nitro-N-nitroso guanidine further improved the organism’s ability to utilize POME for extracellular lipase production.
Figure 6. Lipase activity (U/ml) of strains treated with N-methyl-N’nitro-N-nitroso guanidine during fermentation of POME. Values are means ± standard error of duplicate measurements. NN30 – NN180 are selected strain exposed to N-methyl-N’nitro-N-nitroso guanidine at specified time (min) while wild without exposure.

4. Discussion

In this investigation, POME was chosen as a potential substrate for lipase production. The utilization of the substrate is due to its cheap availability [18]. The composition of medium and mutation of microbial cells affect the growth of microorganisms which invariably affect enzyme production [23]. For maximum utilization of microorganisms, focus should be on strain improvement through the use of mutagenic agents [13]. Genetic variation is believed to be an ideal process for optimization of metabolite production. In this study, the fungal growth for all the treatments continued to rise consistently till day 8. However, some of the mutants performed poorer than the parent wild strain. Reason could be due to the random nature of chemical mutagenesis. Mutant strain NA50 was observed to produced maximum yield of fungal growth and lipase production using POME confirmed the strain to be a suitable mutant to carry out metabolic activity in the medium [9].

The 50 min exposure time of nitrous acid applied to A. niger spores is higher than the timing for the less than 1% survival rate isolate [11]. They reported that A. japonicus subjected to HNO2 treatment produced mutant isolates AHN1 to AHN6, with highest lipase activity in 30s and 45s respectively. The pH continued to decrease even for the parent isolates but isolate NA50 had the lowest pH (3.13) compared to the control (pH 3.31) and other mutants (data not shown). This goes a long way to prove that mutant isolate NA50 is better adapted to metabolism in POME than the other mutants and parent strain. It was observed that the parent strain yielded more free fatty acids than some of the mutants (NA10, NA20 and NA40) despite the fact that these mutants utilized more sugar compared to it. This suggests that the isolates may have produced other metabolites which affected its lipase production reducing the free fatty acids yield.

The maximum of free fatty acid and lipase activity was observed with the mutant strain NA50. This may be due to the fact that HNO2 possibly changed the structure of DNA. The structural alteration in DNA was associated with the activity of the enzyme, probably promoting mycelial growth [24]. Mutant strains from the treatment of nitrous acid showed an improvement in the enzyme production. This may be due to the alteration in the DNA structure by replacing the amino group with hydroxyl group. Nitrous acid is known to encourage deamination of cytosine to uracil and adenine to hypoxanthine. As such, it changes the DNA structure [12]. Compared to the wild strain, isolate NA50 had a 36.94% improvement in lipase activity over it. This result is similar to the report by Mala et al. [13] where A. niger have been subjected to HNO2 treatments showed a 33.3% and 39.1% increase in lipase activity over the parent strain after 60mins of exposure to HNO2. Karanam and Medicherla, [11] subjected A. japonicus to UV irradiation first before subjecting it to HNO2 treatment. They reported the lipase yield of the best HNO2 mutant (AHN3) as 39% higher than UV strain (AUV3) and 77% higher than the parent strain. All these results serve as a pointer to the fact that HNO2 treatment improves lipase production by isolates exposed to it. Mutant strain NA50 was selected for further strain improvement procedure using N-methyl-N’nitro-N-nitroso guanidine (NTG).

Strain improvement through mutation using N-methyl-N’nitro-N-nitroso guanidine (NTG) is known to be an inducer of enzyme production like lipase [25] which is also an optimization parameter for a cost effective lipase production (Mala et al., 2001). High yield of fungal growth was obtained with different strains however, mutant strain NN120 had the highest growth on day 8. N-methyl-N’nitro-N-nitroso guanidine (NTG) is considered to be a very
The NTG treatment of the isolates improved the yield of free fatty acids by the mutants over the control (NA50 strain). Free fatty acid amount present in a medium containing oil determine the extent of lipase activity by the enzyme produced [26, 27]. High FFA content confirmed the production of lipase enzyme that is converting the lipid/fatty acid component in the medium to FFA [9]. High lipase activity also increased the amount of FFA. The mutant strains caused an increase in the FFA than the control (NA50 strain). This is due to the increased metabolic activity of the strains. This implies that there is a variation in the ability of mutants to produce FFA when treated with the mutagen at different duration [26]. This pattern is expected to be observed in the lipase activities of the mutant strains.

Mutant strain (NN120) produced had the highest lipase activity than other strains. Possible over production of microbial lipase could be obtained by inducing mutagenesis. The effect of NTG as resulted from the possible changes in the promoter zones of the coding genes for the enzyme [12]. NTG may deregulate the transcription of mRNA corresponding to the enzyme leading to increment in production [28]. The mutant NN120 being the best NTG strain had a 22.65% improvement in lipase activity compared to the control (NA50) strain and a 70.63% improvement in its lipase activity compared to the wild strain A. niger. Bapiraju et al. [16] reported that NTG mutant strains with 120s and 150s exposure showed a 33% increase in lipase activity compared to the UV irradiated parent strain and a 99% increase in lipase activity compared to the wild strain. Caob and Zhangara, [29] reported an increase in lipase production of 3.25 fold by using a Pseudomonas mutant of UV and NTG. Also, a 200% increase in lipase yield by Aspergillus niger mutant of UV, HNO3 and NTG was reported by Ellaiah et al. [30]. Karanam and Medicherla, [11] reported a 56% increase in lipase activity by NTG mutant strain over the HNO3 parent strain, 61% improvement in lipase activity over the UV strain and a 120% increase in lipase activity over the wild strain. Overall repeated mutation procedure using different chemical mutagens has proven to increase the chances of obtaining more robust and versatile industrial strains of microbes.

5. Conclusion

Improvement of fungal strains for increased and enhanced production of industrial products has been the hallmark of all commercial bioprocessing techniques. These improved strains can reduce cost efficiency of the processes and increased product yield. Results from this study prove that nitric acid (HNO3) and N-methyl-N’nitro-N-nitroso guanidine (NTG) are effective mutagens in strain improvement to enhanced lipase production by A. niger. It is hoped that the high yielding mutant strain (NN120) of A. niger can be exploited commercially for large-scale industrial production of lipase. This study contributes to the development of potent mutants and to the establishment of a novel and economic technique for bioconversion of POME to lipase, thereby reducing pollution hazards.

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Conflict of Interest

The authors declare no conflict of interest in this work.

References


