https://dx.doi.org/10.4314/ijs.v23i1.1

Ife Journal of Science vol. 23, no. 1 (2021)

THE INFLUENCE OF NITROGEN SUPPLEMENTATION ON LIPASE PRODUCTION BY *Aspergillus niger* USING PALM OIL MILL EFFLUENT

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(Received: 15th July, 2020; Accepted: 8th December, 2020)

ABSTRACT

Palm Oil Mill Effluent (POME) is one of the highly polluting materials to the environment hence, utilization of this waste to produce useful industrial enzyme is a welcome development. The influence of different nitrogen supplementation using POME in lipase production by *Aspergillus niger* via fermentation was investigated. POME concentration with distilled water in the following ratios: 1:1, 1:2, 1:3, 1:4 and 1:5 were evaluated for the production of lipase via fermentation. The nitrogen supplements employed in POME media were potassium nitrate (KNO_3), ammonium sulphate ($(NH_4)_2SO_4$), ammonium nitrate (NH_4NO_3), urea and sodium nitrate ($NaNO_3$). The parameters analysed using standard procedures were fungal biomass, lipase activity and pH. From the result, POME ratio 1:1 had the highest fungal growth and lipase activity of $8.61 \pm 0.35 \times 10^6$ g/L and 14.95± 0.63 U/ml respectively. Among the various supplemented nitrogen sources in the POME media, ammonium sulphate supplemented medium had the highest biomass yield and lipase activity of 13.96 ± 0.38 g/L and 22.86 ± 0.23 U/ml respectively. The least biomass yield and lipase activity were from potassium nitrate supplemented medium with values of 12.09 ± 0.04 g/L and 15.02 ± 0.71 U/ml respectively. Fungal biomass and lipase activity from ammonium sulphate were statistically significant when compared to other nitrogen sources ($p < 0.05$). Thus, findings from the study revealed that ammonium sulphate is the best choice of nitrogen supplementation medium for utilization of POME to produce lipase.

Keywords: Nitrogen sources, Lipase, POME, *Aspergillus niger*, Fermentation

INTRODUCTION

The palm oil industry is a major agro-based enterprise in the world contributing towards economic growth of the producing countries (Prasertsan and Binmaeil, 2018). The economic importance includes several applications of its by-products, such as cooking oil, special fats, margarines, soaps, detergents, cosmetics, toothpastes, candles, lubricants, biofuels and electric power (Verla *et al.*, 2014; Rodriguez-Mateus *et al.*, 2016). It is estimated that 1 hectare of palm plant produces 10 - 35 tonnes of fresh fruit bunch per year resulting in 16 million tonnes of palm oil production annually (Ohimain *et al.*, 2013; Rodriguez-Mateus *et al.*, 2016). Malaysia, Indonesia, Nigeria, India, Thailand and Colombia are the largest palm oil producers in the world (Imandi *et al.*, 2010; Iwuagwu and Ugwuanyi, 2014). For every 1 tonne of palm oil produced during its processing, about 5.0 – 7.5 tonnes of palm oil mill effluent (POME) is generated (Okwute and Isu, 2007). Much of this waste are usually not treated or may be improperly treated and often cause serious ecological damage when discharged due to its composition (Madaki and Seng, 2013; Bala *et al.*, 2014).

The POME is an acidic, thick, brownish, viscous and voluminous colloidal suspension rich in organic matters (Madaki and Seng, 2013; Eno *et al.*, 2017). The uncontrolled disposal of these wastes into water bodies may produce important effects such as an alteration of pH, an increase in the organic matter, biological oxygen demand (BOD), chemical oxygen demand (COD), eutrophication and toxic compounds (Edward *et al.*, 2015; Esiegbuya *et al.*, 2016). Thus, utilization of POME as cheap available substrate for useful enzyme (lipase) production, will help in saving money and solve environmental challenges caused by its inappropriate disposal (Hermansyah *et al.*, 2018).

POME has great potential as substrate for the production of useful metabolites due to its high organic content (Wu *et al.*, 2006; Iwuagwu and Ugwuanyu, 2014) and because it is generated in vast amounts throughout the year, particularly in palm oil producing countries such as Nigeria (Edward *et al.*, 2015). Several studies have shown that it is an excellent medium for the cultivation of fungi for the production of microbial enzymes

(Nwuche *et al*., 2013; Oshoma *et al*., 2018). POME contains considerable amounts of oil and grease which makes it a good substrate for microbial lipase production. Lipases (EC 3.1.1.3) are a group of enzymes that catalyse the hydrolysis of glycerides and other fatty acid esters under aqueous conditions and the synthesis of esters in organic solvents (Shiraga *et al*., 2005; Geoffry and Achur, 2018). They are also involved in a wide range of conversion reactions that include interesterification, esterification, alcoholysis, acidolysis, transesterification and aminolysis in non-aqueous media (Savitha *et al*., 2007; Geoffry and Achur, 2018). Various applications of lipases include organic syntheses, hydrolysis of fats and oils, modification of fats, flavour enhancement in food processing, resolution of racemic mixtures, and chemical analyses (Kumar and Ray 2014; Sumarsih *et al*., 2019). Microbial lipases have been given special industrial consideration due to their stability, selectivity, and broad substrate specificity (Oshoma *et al*., 2018). This is due to their catalytic activities, high yields, ease of genetic manipulation, absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media (Nwuche and Ogbonna, 2011).

Major reports on microbial lipase have been on its production from fungi sources because they are usually excreted extracellularly, facilitating extraction from fermentation media (Oshoma *et al*., 2018). Filamentous fungi are recognized as the best lipase producers and preferred sources due to low cost of extraction, thermal and pH stability, and activity in organic solvents (Ghosh *et al*., 1996). The primary fungal producers of commercial lipases belong to the genera *Aspergillus, Mucor*, *Candida*, *Rhizopus* and *Humicola* species (Eno *et al*., 2017; Sumarsih *et al*., 2019).

Optimum concentrations of substrates and nitrogen sources supplementation are both economical and environmental-friendly for efficient production of metabolites by microorganism (Sridevi and Ashishi, 2016). Increased biomass is favoured in media with nitrogen supplied to the organism (Nwuche *et al*., 2013). Thus, identifying major scale-up parameters during fungi cultivation for the production of useful metabolites is a key towards sustainable enzyme production. Therefore, the influence of different nitrogen supplementation using POME in lipase production by *Aspergillus niger* was investigated, since nitrogen compounds have been found to be useful macronutrient for nucleic acids and protein biosynthesis.

MATERIALS AND METHODS

Sample Collection

Palm Oil Mill Effluent (POME) samples were collected from the Oil Mill Division of the Nigerian Institute for Oil Palm Research (NIFOR) Benin City, Edo State (Geographical coordinates of 6° 30' 0" North, 6° 12' 0" East) and was taken immediately to the laboratory.

Fungal Isolation and Identification

Fungal strain, *Aspergillus niger,* used in this study was isolated from soil contaminated with POME based on standard methods of Barnett and Hunter (1972), and Larone (1986). The number of spores was counted using a Neubauer haemocytometer (depth 0.1 mm, area 0.0025 mm^2, Marienfield, Germany) and inoculum size of 10^6 spores/ml of each fungal spores was used to inoculate all the media respectively.

Fermentation Process

Raw POME used as substrate for fermentation was passed through a hand sieve of 0.5 mm pore size to eliminate coarse solids such as broken shells, kernels and plant fibers. The fermentation was carried out using a submerged fermentation process. The effect of POME concentration on lipase activity was investigated by diluting the POME with distilled water in the ratios of 1:1, 1:2, 1:3, 1:4 and 1:5. From the various stock ratios, 100 ml was dispensed into the labelled 250 ml Erlenmeyer flasks, sealed with cotton wool and foil paper and thereafter autoclaved at 121 °C for 15 min. After sterilization each labelled flask containing 100 ml of POME of various ratios were aseptically inoculated with spores' suspension of *A. niger* (10^6 spores/ml). All inoculated flasks were fermented for a duration of 8 d on a rotary shaker at 120 rpm. Samples were taken at every 2 d interval for evaluation of fungal biomass, lipase activity and pH changes of medium.

Effect of Nitrogen Supplementation on POME

The POME medium that gave the highest yield of lipase was supplemented with different nitrogen sources. The modified method of Oshoma *et al.* (2017) was used. The nitrogen sources employed were ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3), urea, ammonium sulphate ($(NH_4)_2SO_4$), and sodium nitrate ($NaNO_3$) for each to supply 0.50 gN/L in POME medium. A 100 ml of the broth was transferred into 250 ml Erlenmeyer flasks. The samples prepared, prepared in triplicates, were autoclaved at 121 °C for 15 min. The flasks containing the various nitrogen supplemented media were inoculated with 500 µl spores' suspension of *A. niger,* while the control medium was without nitrogen source. The media were allowed to undergo fermentation on an orbital shaker at 120 rpm at temperature of 28 ± 2 °C followed by determination of fungal biomass, lipase activity and pH changes of medium after 6 d of fermentation.

Analytical Methods

Fungal growth was determined using Whatman's no 1 filter paper for filtration. The filtered mycelium was washed with sterile distilled water and dried at 105 °C for 24 h and weighed. Lipase activity was measured by modification of the titrimetric assay method of Pignede *et al.* (2000). The substrate (primary) emulsion was prepared with appropriate measure of 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. The supernatant of the cell free 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 min at 37 °C with shaking (120 rpm). A 4 ml acetone-ethanol (1:1 v/v) solution was added to stop the reaction and 2 drops of 0.09% phenolphthalein as indicator. Lipase activity was determined by titration with 50 mM NaOH. All lipase activity assays were performed in triplicate. One unit of lipase was defined as the amount of enzyme that catalyzes the release of 1 µmol of fatty acids per minute at 37 °C. Changes in pH were determined using pH meter (ITT Analytics, model 314P).

Statistical Analysis

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

RESULTS

Fungal biomass during the fermentation of varying ratios of POME is shown in figure 1. The highest fungal biomass was recorded on day 6 in all POME ratios and then there was a decrease afterward. At the growth peak (day 6), the highest fungal biomass was recorded in POME ratio 1:1 (8.61±0.35 g/L) while the least was POME ratio 1:5 (3.71±0.12 g/L). Statistically, there was significant difference when comparing fungal biomass of POME ratio 1:1 and other ratios ($p< 0.05$) after 6 d of fermentation.

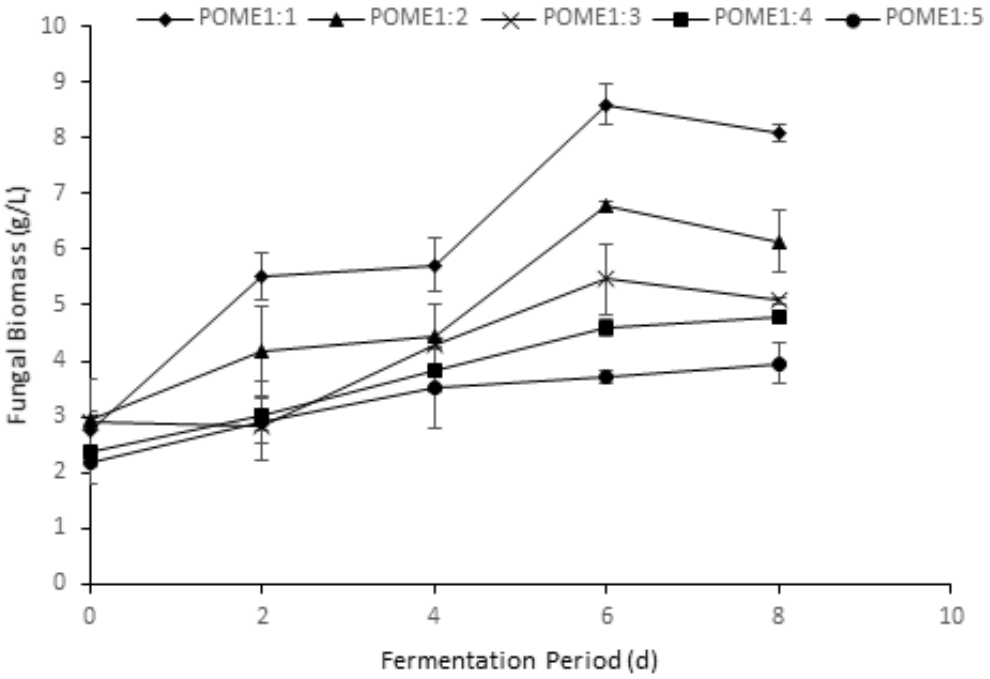

Figure 1: Fungal Biomass during the Fermentation of varying POME Ratios on a Time Course Basis. Values are means ± standard deviation of triplicate measurements.

The lipase activities during the fermentation of varying POME ratios are shown in figure 2. The highest lipase activity was recorded on day 6 in all POME ratios and there was a decrease afterward. On day 6, the highest lipase activity of 14.95±0.63 U/ml was from POME ratio 1:1 while the least 9.35±0.71 U/ml was POME ratio 1:4. Statistically, comparing lipase activity of POME ratio 1:1 to other ratios showed a significant difference ($p<$ 0.05).

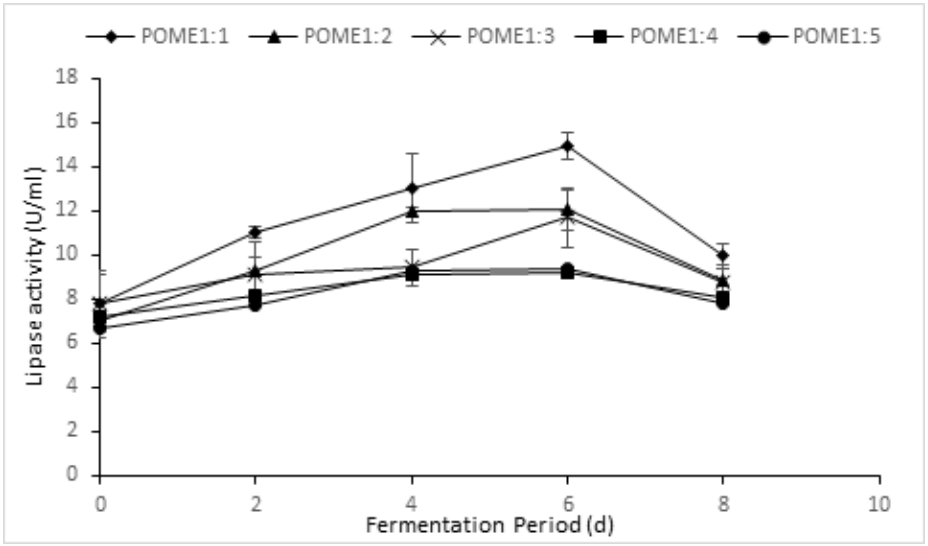

Figure 2: Lipase Activity (U/ml) of *A. niger* during the Fermentation of varying POME Ratios on a Time Course Basis. Values are means ± standard deviation of triplicate measurements.

Table 1 showed changes in pH during the fermentation of varying POME ratios. The highest pH after 8 d of fermentation was recorded in POME ratio 1:5 while the least was recorded in POME ratio 1:1 with valuesof 4.40±0.00 and 4.15±0.05 respectively.

Table 1: Changes in pH during the Fermentation of varying POME Ratios

POME ratio	Fermentation Period (d)				
	0	2	4	6	8
POME 1:1	5.00±0.05	4.45±0.05	4.35±0.05	4.40±0.00	4.15±0.05
POME 1:2	5.00±0.05	4.40±0.00	4.30±0.00	4.25±0.05	4.30±0.00
POME 1:3	5.00±0.05	4.45±0.05	4.30±0.05	4.30±0.00	4.30±0.00
POME 1:4	5.00±0.15	4.45±0.05	4.25±0.05	4.25±0.05	4.20±0.00
POME 1:5	5.00±0.00	4.45±0.05	4.35±0.05	4.45±0.05	4.40±0.00

The influence of media supplemented with nitrogen sources on *A. niger* growth and lipase production was studied using POME ratio 1:1, which previously showed the highest lipase yield than other ratios investigated. In this study, various nitrogen sources such as ammonium nitrate, ammonium sulphate, sodium nitrate, potassium nitrate, urea and control (without nitrogen source) were investigated. During fermentation, the pH was found to decrease in most of the nitrogen supplemented media (Table 2). The pH value of ammonium sulphate and control media decreased to 4.40 ± 0.07 and 4.40 ± 0.00 respectively on day 6 of fermentation.

Table 2: Changes in pH Values of the different Nitrogen Supplemented Media during Fermentation

Nitrogen Sources	Fermentation Period (d)	
	0	6
Ammonium nitrate	5.00 ± 0.06	4.40 ± 0.07
Ammonium sulphate	5.00 ± 0.06	4.40 ± 0.06
Sodium nitrate	5.00 ± 0.06	4.40 ± 0.14
Potassium nitrate	5.00 ± 0.08	4.45 ± 0.07
Urea	5.00 ± 0.11	4.45 ± 0.07
Control	5.00 ± 0.07	4.40 ± 0.00

Fungal growth in the different nitrogen supplemented media during fermentation is shown in figure 3. All the nitrogen sources and control (without nitrogen) showed increase in growth from 0 to 6 d. The highest fungal growth was 13.96 ±0.38 g/L for ammonium sulphate medium followed by ammonium nitrate medium and the least was control (without nitrogen) medium (8.61 ± 0.04 g/L) on day 6.

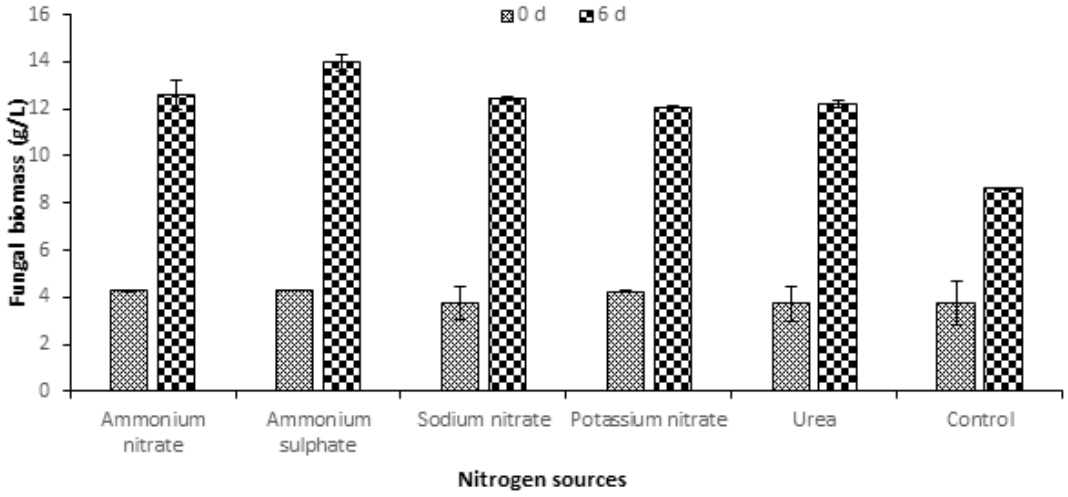

Figure 3: Biomass Yield of *A. niger* in the Various Nitrogen Sources Media in Submerged Fermentation at 120 rpm for 6 days

The effect of media supplemented with nitrogen sources on *A. niger* lipase production was studied using POME. The lipase activity of the investigated nitrogen supplemented media during the fermentation period is shown in figure 4. The highest lipase activity of 22.86 ± 0.23 U/ml was from $(NH_4)_2SO_4$ supplemented medium followed by NH_4NO_3 medium (20.78 ± 0.35 U/ml) while the least 14.95 ± 1.06 U/ml was from control medium without nitrogen supplementation. Statistically, comparing lipase activity of $(NH_4)_2SO_4$ supplemented medium to other media showed a significant difference ($p < 0.05$).

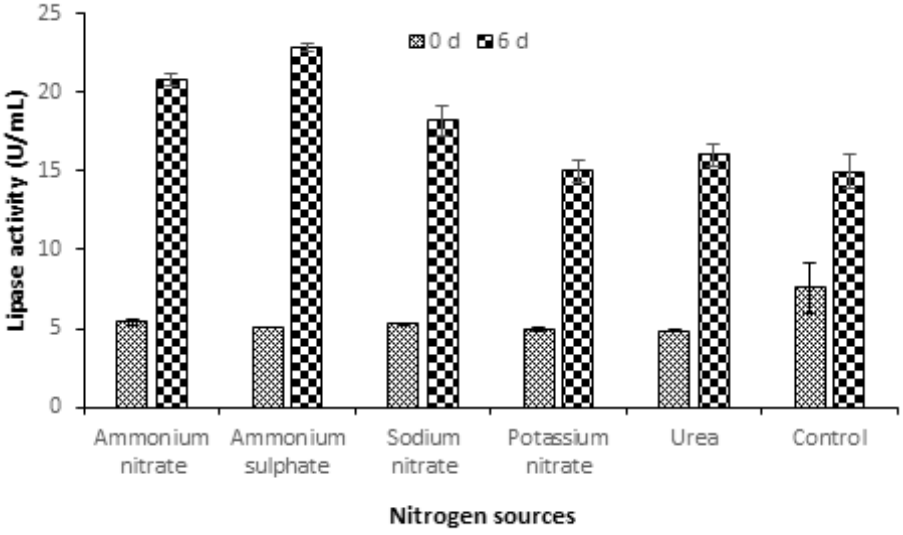

Figure 4: Lipase Activity of *A. niger* in Various Nitrogen Supplemented POME Media in Submerged Fermentation at 120 rpm for 6 days

DISCUSSION

The growth and multiplication of microbial species on any medium is often considered as the first step towards its utilization (Molla *et al*., 2002). The increase of *A. niger* biomass from POME as observed in this study could be as a result of its ability to metabolize and utilize POME as carbon and energy sources for growth and other metabolic reactions (Costa *et al*., 2017). The concentration of substrate for microbial fermentation should be available in sufficient concentrations (Wu *et al*., 2006). Biomass yield across the varying POME ratios showed significant increase in cell mass with the highest fungal biomass recorded in POME ratio 1:1 on day 6 indicating this ratio had enough nutrient compositions. In addition, this ratio (POME 1:1) is an indication that it contains metabolizable nutrient sufficient to support fungal growth (Eno *et al*., 2017).

To overcome these substrate-associated challenges in this present study, lipase yield was determined in all the five ratios of POME (1:1, 1:2, 1:3, 1:4 and 1:5) inoculated with *A. niger*. The highest lipase activity was recorded in POME ratio 1:1 while the least lipase activity was recorded in POME ratio 1:5 indicating the inductive effect of POME and its concentration dependence. This finding suggests that POME ratio 1:1 is most suitable for lipase production as other POME ratios in this study resulted in lower lipase yield. The finding of this study is in agreement with that of Nwuche *et al.* (2013) who reported that POME ratio 1:1 inoculated with *A. terreus* had the highest lipase activity. Lima *et al.* (2003) reported that low lipase activities in higher POME concentrations and ratios as observed in this study may have resulted from lipase biosynthesis inhibition which may be due to poor oxygen transfer into the medium. Elibol and Ozer (2000) noted that low oxygen supplies during fermentation impacts negatively on fungal metabolism and consequently the production of lipases. The low lipase activity reported in other substrate composition may be caused by poor contact

between the organism and the oil components of the medium. Salihu *et al.* (2011) reported that the highest lipase activity was observed at 0.5% POME and further increase in POME concentration showed a significant inhibitory effect on extracellular lipase production. Maximum growth and lipase production by *A. niger* is enhanced due to availability of nutrients such as sugars that are metabolised by the fungi (Mukhtar *et al*., 2015).

The pH is important for the optimal physiological performance of the fungi and nutrient transportation to various cell membranes (Mukesh *et al*., 2012). Optimal lipase production by fungal isolates usually occurs under acidic pH. Kumar and Ray (2014) showed that lipase production by *A. niger* had a good stability at pH 4.0 as compared to other investigated fungal isolates. Similar pattern was also observed in *Rhizopus arrhizus* (MTCC 2233) by Rajendran and Thangavelu (2009), where decrease in pH value was attributed to organic acid production during the enzyme production. Results from this study showed that all the media had acidic pH on day 6 signifying that its metabolism was highest in the fermentation setups. These organic acids such as free fatty acid, produced by *A. niger* could possibly have been the cause of the steady decrease in pH of the medium (Costa *et al.,* 2017).

Nitrogen is the main building block of proteins (enzyme) and is one of the main constituents of living organisms. Supplementation of nitrogen is used to optimize the nitrogen requirement of most microbial species especially fungi (Oshoma *et al*., 2017). The ability of the various nitrogen sources mostly ammonium sulphate to support POME as a substrate to increase fungal growth confirmed the substrate to be a suitable medium for fungal growth and metabolite production (Costa *et al*., 2017). The highest growth obtained from ammonium sulphate medium showed it to be the best nitrogen source for POME utilization. Boonchaidung *et al*. (2013) reported that supplementation of media with nitrogen sources enhanced fungal growth.

Oil from POME can be used as inducers for lipase production and as a sole carbon source in the medium for the fungi to utilize (Sethi *et al*., 2015). Media supplementation by nitrogen sources is known to enhance enzyme production such as lipase (George-Okafor *et al*., 2013) which is one of the optimization parameters for a cost effective enzyme production (Mishra *et al*., 2016). Commonly used inorganic nitrogen sources are $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl. High yields of lipase activities were obtained with the different nitrogen sources and $(NH_4)_2SO_4$ gave the highest lipase activity. POME is poor in some chemical composition required for growth, such as protein (Linde *et al*., 2006). Supplementation of media with nitrogen would enhance fungal growth and worthy of note is ammonium salts which are stimulators of microbial growth and lipase production. Several authors had reported that medium supplemented with $(NH_4)_2SO_4$ as nitrogen source gave the highest yield of biomass and lipase by *A. niger* in submerged fermentation (George-Okafor *et al*., 2013; Mishra *et al*., 2016; Oshoma *et al*., 2017). Thus, confirming our findings that medium supplemented with $(NH_4)_2SO_4$ enhanced fungal growth which in turn, increased biomass cropped and lipase activity in the POME medium.

CONCLUSION

This investigation suggests that POME could be utilized as a promising substrate for *A. niger* growth and production of lipase rather than allowing it to cause environmental pollution. Supplementation of the POME medium with ammonium sulphate is found to be beneficial for enhancing enzyme production yield.

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