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Original Research Article

PRODUCTION OF LIPASE FROM PALM OIL MILL EFFLUENT USING FUNGAL ISOLATES

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ABSTRACT

Palm oil mill effluent (POME) management has been a major environmental issue in palm oil producing nations of the world like Nigeria. However, microorganisms such as fungi can utilise these effluents for the production of useful industrial enzymes via fermentation. Fungal isolates from POME contaminated soil were evaluated for the production of lipase via fermentation of the effluent. Fungal growth, free fatty acid percentage, lipase activity, pH and reducing sugars were analyzed in the course of fermentation using standard procedures. Fungal isolates from soil contaminated with POME were identified as Aspergillus niger, Aspergillus tamarii, Penicillium notatun and Saccharomyces cereviseae. From the results, A. niger had the highest fungal growth and lipase activity of 14.00 ± 0.55 x 10^6 cfu/ml and 8.50 ± 0.12 U/ml respectively while the least fungal growth and lipase activity of 10.30 ± 1.00 x 10^6 cfu/ml and 4.33 ± 0.05 U/ml respectively were for A. tamarii. Fungal growth and lipase activity for A. niger were statistically significant when compared to other fungal isolates ($p < 0.05$). Thus, the investigation revealed that A. niger can be the choice of fungal isolate for bioconversion of POME for lipase production.

1. INTRODUCTION

Regulations to ensure the safety of aquatic environment by World Health Organization (WHO) and the Federal Environmental Protection Agency (FEPA) have not been so effective in Nigeria (Edward *et al.*, 2015). This is partly because of lack of Government enforcement as well as negligence on the part of the citizenry. Palm oil mill effluent (POME) generated from palm oil mill industries especially in Southern Nigeria is one of the most common pollutants indiscriminately dumped in the Nigerian aquatic ecosystems (Abid Baig *et al.* 2003). It is estimated that 5.0 – 7.5 tonnes of POME is generated from every 1 tonne of palm oil produced during its processing (Ahmad *et al.*, 2008). This raw effluent causes pollution of waterways as well as, death of aquatic lives due to its low pH, high BOD and colloidal nature (Esiegbuya *et*

al., 2016). Environmental impact of indiscriminate dumping of POME can result in offensive odour emissions, lack of access to good water and environmental health challenges (Igwe and Onyegbado 2007). However, the raw effluent is known to contain high organic matter that are natural substrates for microorganism (Imandi *et al*., 2010). The utilization of this cheap readily available substrate to useful product such as microbial enzyme is a welcome biotechnological development and will reduce operation cost of production (Imandi *et al*., 2010; Iwuagwu and Ugwuanyu 2014). Also, bioconversion of this cheap available substrate to useful enzymes such as lipase, will help in reducing pollution caused by the waste (Bacha *et al*., 2011)

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are widely produced in nature by plants, animals and several microorganisms, but only microbial lipases are commercially significant (Sharma *et al*., 2001). The term "lipolytic enzymes" refers to lipases and carboxylic ester hydrolases, and the former differs from the latter due to their ability to act in insoluble esters. Lipases act mainly in emulsified substrates with long fatty acid chains (triglycerides), while the carboxylic ester hydrolases act in soluble esters with relatively short fatty acid chains (Esiegbuya *et al*., 2016). This is the reason why lipases, in contrast to other esterases, need an oil–water interface for optimum activity (Benjamin and Pandey, 1997). Commercially valuable lipases are mostly obtained from microorganisms that produce a wide variety of extracellular lipases (Akimoto, 1999).

Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids unlike esterases (Sharma *et al*. 2001) and have emerged as one of the leading biocatalysts required in bio-industries with potentials economic enhancement (Joseph *et al*. 2008). Microbial lipases have been given special industrial consideration due to their stability, selectivity, and broad substrate specificity (Dutra *et al*. 2008). Various applications of lipases include organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses (Sharma *et al*. 2001).

Plants, animals and microorganisms can be a natural sources of lipase production however, more attention is currently given to microbial lipase because of their technical and economic advantages, lipase from them are more stable as compared to plants and animals (Lau *et al*., 2011; Sundar *et al*., 2012).

Among the microorganisms used for production of lipases, fungi are the most utilized for industrial applications because they are one of the sources for lipase biosynthesis (Iftikhar *et al*., 2012). Fungal species such as *Aspergillus*, *Rhizopus*, *Mucor* and *Penicillum* spp are considered to be good producers of commercially useful lipase and strive very well on POME (Eno *et al*., 2017). In this study, POME was exploited for lipase production due to its availability and low cost using lipolytic fungi. On this premise, the study was aimed at the investigation of POME as alternative raw material (substrate) for cost effective fungal lipase production.

2. METHODOLOGY

2.1. Sample Collection and Preparation

Palm Oil Mill Effluent (POME) samples 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 cleaned and rinsed with 70% ethanol and sterile distilled water.

2.2. Inocula Preparation and Size

Fungal strains used in this study were isolated from soil contaminated with POME based on cultural and microscopy characterization following standard methods of Barnet and Hunter (1972) and Larone (1986) and maintained on potato dextrose agar (PDA) slant and stored at 4 ^{o}C. The different fungal inocula were

prepared from subcultured fungal isolates on potato dextrose agar (PDA) plates that were incubated for 5 days. The fungal isolates cultured plate was flooded with 10 mL of sterile 1% $^{v}/_{v}$ tween 80 solution to dislodge the spores from the hyphae. The solutions with spores were filtered with sterile muslin cloth to remove any hyphal fragments present (Ikenebomeh and Chikwendu 1997). The number of spores were counted using a haemocytometer and inoculum size of 10^6 spore/mL of each fungal spore was used to inoculate all the media respectively.

2.3. 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^{o}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 of the four (4) identified fungal isolates respectively with inoculum size of 10^6 spore/mL while the control media was uninoculated. The media were left to ferment on an orbital shaker at 120 rpm at temperature of 28± 2 ^{o}C followed by determination of fungal growth, lipase activity, FFA, pH of medium and reducing sugars at every 2 days interval for 8 days.

2.4. 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 ^{o}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.* (2000). 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 50mM phosphate buffer (pH 7.0 (Na_2HPO_4-KH_2PO_4)) before incubating for 20 mins at 37^{o}C with shaking (120 rpm). The reaction was stopped with 4 mL of acetone-ethanol (1:1 v/v) containing 2-3drops 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 ^{o}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 (Onilude *et al*., 2010). The Free Fatty Acid was calculated using modified method of Kanimozhi *et al.* (2011).

Determination of pH was through the use of pH meter (3305 Jenway, England). Estimation of reducing sugars in the samples was carried out according to the method described by was A. O. A. C. (2005). Five grams (5 g) of NaOH was added to 400 mL of distilled water and by continuous stirring 100 g of sodium potassium tartarate was added. After that 250 mg of sodium sulphite, 5 g of DNS (3.5-dinitrosalicylic acid) and 1 mL of phenol were added to the solution. Then it was filtered and kept under constant stirring in dark room to avoid the exposure of DNS to light. The reducing sugar content of the fermentation broth was assayed by adding 2 ml of DNS reagents to 1 mL of the sample. The mixture was then heated in boiling water for 5 min and then cooled under running tap water. The absorbance at 540 nm of the resulting coloured solution (slight brown) was read in a spectrophotometer against a blank, prepared by substituting the hydrolyzed sample with distilled water. The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

2.5. 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 AND DISCUSSION

The isolated fungal culture obtained from soil contaminated with POME were related mainly to *Saccharomyces cereviseae*, *Aspergillus tamarii,* Aspergillus *niger* and *Penicillium notatun* as shown in Table 1. Similar fungal isolates were found in POME impacted soil. Eno *et al.* (2017) also reported that *Aspergillus* species were most abundant fungi identified from the soil.

Table 1: Cultural and microscopic characteristics of the fungal isolates from soil contaminated with POME.

Characteristics	F1	F2	F3	F4
Cultural	Creamish colony with reverse side cream	Dark greenish colony with reverse side colourless	Black fluffy colony with reverse side yellow	Greenish colony with white periphery
Microscopic				
Nature of hyphae	Pseudohyphae	Septate	Septate	Septate
Colour of spore	Cream	Yellow	Brown	Greenish
Type of spore	Chlamydospore	Conidiospre	Conidiospore	Conidiospore
Appearance of special structure	Budding	Foot cells	Foot cells	Brush-like conidia
Possible isolates	*Saccharomyces cereviseae*	*Aspergillus tamarii*	*Aspergillus niger*	*Penicillium notatun*

The fungal isolates were screened quantitatively for lipase production through submerged fermentation in POME broth. Fungal growth during the fermentation is shown in Figure 1. All the isolates showed increase in growth from 0 to 8 days. The highest fungal growth was 14.0±0.55 x 10^6 cfu/mL for *A. niger* followed by *S. cerevisea,* and the least was *P. notatum* (10.30 ± 1.00 x 10^6 cfu/mL) on day 8. Statistically, there was significant difference when comparing growth of *A. niger* and *S. cerevisea* ($p< 0.05$). There was no growth in the control uninnoculated POME sample.

Growth and multiplication of microorganisms on any substrate is often considered as the first step towards its bioconversion (Molla *et al.,* 2002). The ability of the various fungal isolates to utilize POME as a substrate to increase cell growth confirmed the substrate to be a suitable medium for fungal growth and metabolite production (Costa *et al*., 2017). The highest growth obtained by *A. niger* showed it to be the best fungal isolate for POME utilization. This is consistent with the report of Nwuche *et al.* (2013), who obtained maximum *A. nige*r growth when cultured in POME. It was discovered that after 8 d of incubation, *A. niger* had the highest fungal cell growth recorded followed by *S. cerevisea, A. tamarii* and *P. notatum.* This could imply that *A. niger* was more adapted to POME compared to the other organisms and hence it was most efficient in utilizing it for cell growth.

The percentage free fatty acid yield during the fermentation is shown in Figure 2. The highest yield of 25.01±1.80 % was from *A. niger* followed by *S. cerevisea*, *P. notatum* and *A. tamarii* respectively on day 8. There was no free fatty acid yield in the uninoculated POME sample (control). The lipase activities of the fungal isolates during the fermentation is shown in Figure 3. The highest lipase activity of 8.50 ± 0.12 U/mL was from *A. niger* while the least 4.33 ± 0.05 U/mL was *A. tamarii* on day 8. The uninoculated POME showed no increase in lipase activity. Statistically, comparing lipase activity of *A. niger* to *S. cerevisea* showed a significant difference ($p< 0.05$).

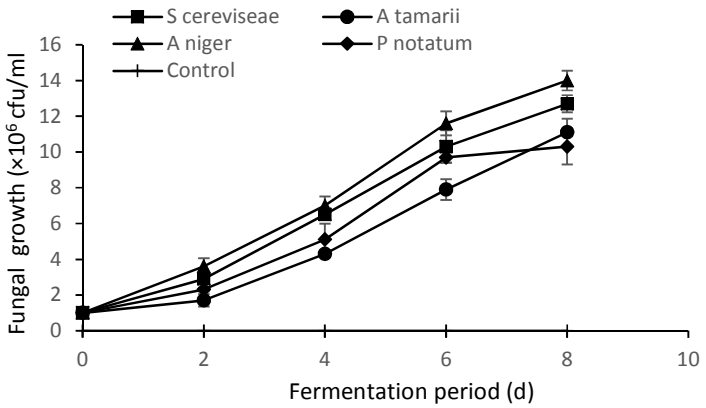

Figure 1: Fungal growth of isolates during fermentation of POME on a time course basis.

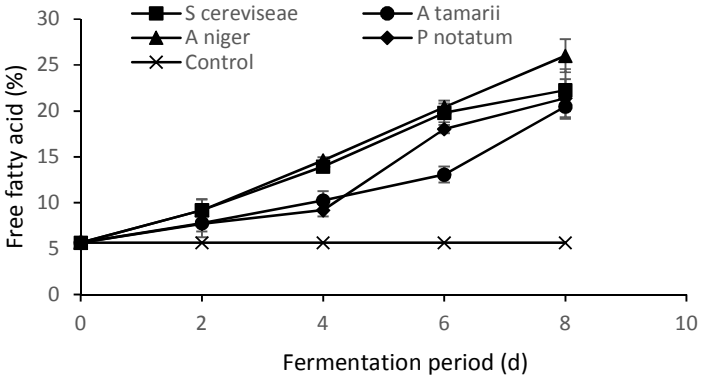

Figure 2: Free fatty acid (% FFA) of fungal isolates during POME fermentation on a time course basis. Values are means ± standard error of duplicate measurements.

The result showed variability in the % FFA with respect to the different fungal isolates. This is a fact that various isolates possess different ability to produce the enzyme lipase. The amount of free fatty acid in the medium is a measure to which glyceride in the oil has been hydrolysed by the enzyme lipase and an indication of the degree of lipase activity taking place (Onilude *et al*., 2010; Esiegbuya *et al*., 2016). Among the fungal isolates, *A. niger* was observed to increase its lipolytic activity due to its higher amount of FFA produced. This study agreed with the report of Costa *et al.* (2017) that *A. niger* gave the highest % FFA among the fungal strains screened.

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). Among all the fungal isolates from this study, highest lipase activity was observed in the medium inoculated with *A. niger*, indicating that it can express the extracellular enzyme faster than the other isolates (Mukhtar *et al*., 2015) and *A. niger* is regarded as Generally Recognized as Safe (GRAS) by Food and Drug Adminstration (Kumar and Ray., 2014). The incubation period is an important factor for the production of extracellular lipases by the microorganism (Imandi *et al*., 2010). Maximum lipase

activity was observed on day 8. This confirmed the report of Kumar and Ray (2014) that maximum lipase activity was observed after the 8 d of fermentation by *A. niger* when oily substrates were utilized. This is contrary to Mahadik *et al.* (2002) who reported maximum lipase production by *A. niger* on the day. Shirazi *et al.* (1998), posited in their study on extracellular lipase production using *S. cerevisiae* that there was a correlation between maximum production of extracellular lipase and cell mass after 18 h of growth (incubation period). This implies that production of the enzyme is growth-associated and that lipase activity increased with increase in cell mass.

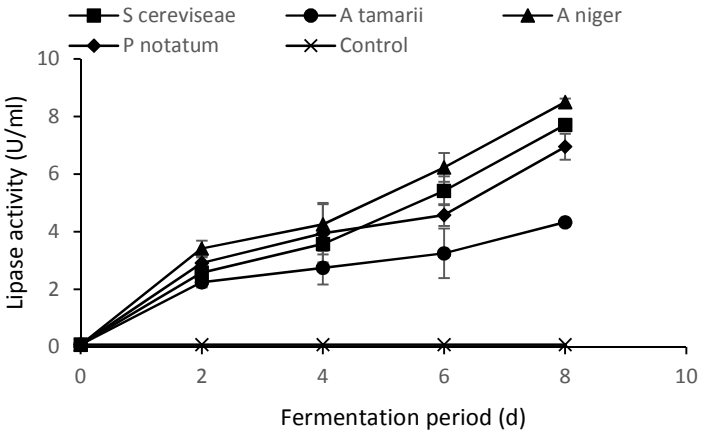

Figure 3: Lipase activity (U/ml) of fungal isolates during POME fermentation on a time course basis. Values are means ± standard error of duplicate measurements

As shown in Table 2, the pH of the medium for all the treatments except the control dropped from 4.50 on 0 d to 3.71, 3.59, 3.67 and 3.31 on the 8 d for *A. tamarii*, *S. cerevisea, P. notatum and A. niger* treated POME samples respectively. Table 3 showed the sugar utilization activities of the fungal treatments in the POME. The reducing sugar on day 0 was 1.355±0.00 g/mL but on day 8 it reduced to 0.727±0.05, 0.622±0.03, 0.570±0.10 and 0.500±0.05 g/mL for *A. tamarii*, *P. notatum, S. cerevisea, and A. niger* respectively. The sugar level in the uninoculated POME sample (control) remained unchanged.

Table 2: pH value of fungal isolates during fermentation of POME

Fungal Isolates	Period of fermentation (d)				
	0	2	4	6	8
Saccharomyces cereviseae	4.50	4.40	4.06	3.84	3.59
Aspergillus tamari	4.50	4.46	4.05	3.99	3.71
Aspergillus niger	4.50	4.43	4.02	3.40	3.31
Penicillium notatum	4.50	4.46	4.02	3.89	3.67
Control	4.50	4.50	4.50	4.50	4.50

Table 3: Reducing sugar (g/mL) of fungal isolates during fermentation of POME

	Period of fermentation (d)				
Fungal Isolates	0	2	4	6	8
Saccharomyces cereviseae	1.35±0.00	1.20±0.05	1.14±0.01	0.85±0.03	0.57±0.1
Aspergillus tamari	1.36±0.00	1.26±0.06	1.08±0.03	0.95±0.04	0.73±0.05
Aspergillus niger	1.35±0.00	1.15±0.03	0.95±0.01	0.75±0.02	0.50±0.05
Penicillium notatum	1.35±0.00	1.07±0.08	0.96±0.04	0.77±0.05	0.62±0.03
Control	1.35±0.00	1.36±0.00	1.35±0.00	1.35±0.00	1.35±0.00

As indices to further determine the best of the four isolates in lipase production, the pH and sugar content of the fermentation medium are important environmental factor which influences production of lipase. The pH

is important for the optimal physiological performance of the fungi and nutrient transportation to various cell membrane (Mukesh *et al.*, 2012). Optimal lipase production by fungal isolates usually occur 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 *A. niger* had the lowest pH on day 8 signifying that its metabolism was highest compared to the other fungi in the different fermentation setups. These organic acids such as free fatty acid, produced by the isolates could possibly have been the cause of the steady decrease in pH of the medium (Costa *et al.,* 2017). Utilization of sugars in the medium by microorganisms influence enzyme production (Mukesh *et al.*, 2012). *A. niger* was found to had lower % reduction of sugars. Maximum growth and lipase production by *A. niger* is enhanced due to availability of nutrients like sugars that are metabolised by the fungi (Mukhtar *et al.*, 2015).

4. CONCLUSION

The Palm Oil Mill Effluent (POME) supported the growth of fungal isolates and lipase production as an evidence that the substrate is been utilized by the isolates. The significant finding is that *A. niger* was found to produce both higher growth and yield of lipase when POME was used as the fermentation medium.

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6. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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