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Effect of Culture Condition on Lipase Production by *Bacillus species* Isolated from Palm Oil Mill Effluent Polluted Soil

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Abstract

The present study investigated the effects of culture conditions on lipase production by pure culture of *Bacillus species* isolated from palm oil mill effluent polluted soil. The assessment was based on the following parameters such as lipidic substrates (carbon sources), nitrogen sources, pH, incubation time and inoculum size. Lipase activity in the presence of lipidic substrates increased in the following order of Palm kernel oil > Groundnut oil cake > Tween 80 > Coconut oil cake and the maximum as well as minimum lipase activity value of 22.78 ± 0.1 and 20.78 ± 0.3 µmol/min were obtained respectively. Yeast extract and near alkaline pH (7.5) were found to be the best nitrogen source and pH for lipase production by the test organism with lipase activities of 6.8 ± 0.4 and 6.7± 0.1 µmol/min respectively. The highest lipase activity values of 21.8 ± 0.4, 18.9 ± 0.1, 19.6 ± 0.2 and 21.1 ± 0.3 µmol/min for Palm kernel oil, Groundnut oil cake, Tween 80 and Coconut oilcake were obtained at 72 h incubation time. The highest and lowest lipase activity of 15.3 ± 0.4 and 9.7 ± 0.1 µmol/min were obtained at inoculum size of optical density of 0.2 and 0.8 respectively. The above results revealed that the palm oil mill effluent medium can be a good source for isolation of lipase producing bacterial strains and modifying the composition of the growth medium will greatly influence high yield of lipase enzyme by the test isolate.

Keywords: lipase, *Bacillus sp.,* production, palm oil effluent, soil.

1. Introduction

Environmental pollution due to crude oil effluents has been one of the major problems faced by the industrialized world today. However, other sources of environmental pollution seemed to be overlooked among which include effluents arising from palm oil mill processing plants. Palm oil mill effluent (POME) is generated from the production of palm oil. It was estimated that during the production of 1 tonne of crude palm oil, more than 2.5 tonnes of POME are produced. Analysis of POME showed that it is acidic, brownish and colloidal suspension with 95 to 96% of water, 0.6 to 0.7% of oil and 2 to 4% total suspended solids (TSS). This can lead to considerable environmental problems by polluting water and destroying aquatic biota if discharged without effective purification (Cheng, Mnif, Hadrich, Abdelkafi and Sayadi,

2010). However, excessive quantities of unpurified POME have been reported to deplete a water body of its oxygen and suffocate aquatic life. Many rivers have been devastated by such discharge as people living downstream are usually affected (Madaki and Seng. 2013). As a result of this, the development of new technologies that emphasize on the detoxification and destruction of the contaminants rather than the conventional approach of disposal has been proposed. The use of microorganisms or microbial process to detoxify and degrade oil effluents is one of the recent innovative technologies gaining ground in the last two decades.

Microorganisms such as bacteria, yeast and fungi are known to secret lipases. Lipase-producing microorganisms are ubiquitous in nature and have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, soil contaminated with oil, etc. Many efforts have been made to isolate lipase producing microorganisms since this enzyme is used in numerous biotechnological processes such as food, leather, cosmetic, detergents and pharmaceutical industries as well as industrial wastes management (Sztajer, Maliszewska and Wieczorek, 1998). The oily environment such as palm oil mill effluent may provide a good environment for isolation of lipase producing microorganisms. Among all the microorganisms, bacterial lipases are more economical and stable and are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat. Bacterial lipases are mostly extracellular being excreted through the external membrane into the culture medium and are greatly influenced by nutritional and physicochemical factors which include pH, nitrogen and carbon sources, inorganic salts, dissolved oxygen concentration, temperature**,** etc. The improvement in culture conditions for the production of lipases is of great interest in as much the cultural conditions do not influence the enzyme properties as well as the enzyme extra to intracellular ratio (Cherif, Mnif, Hadrich, Abdelkafi, and Sayadi, 2011; Puthli, Rathod and Pandit, 2006). The optimum conditions for lipase production by bacteria isolated from POME are yet to be properly described in full detail.

Against these backdrops, the present study was aimed at isolating and assesses the effect of different culture conditions on lipase production by the isolated bacterial strain.

.2. Materials and methods

2.1 Study Area and Sample Collection

Palm oil mill effluent (POME) sample was collected from the discharge tank of palm oil mill processing plant using 5 litre sterile polyethylene containers from Umunoha in Mbaitoli local Government Council of Imo State, Nigeria. Samples were stored in a cooler box and taken to laboratory for isolation of lipase producing organisms under laboratory condition.

2.2 Isolation and Screening of Lipolytic Organisms

One mililitre (1.0 ml) of the palm oil mill effluent sample was transferred into 9.0 ml of physiological saline contained in 20 ml screw capped test tube and shake vigorously. This was subsequently further serially diluted up to 10^6. From this, one tenth (0.1 ml) of the diluted sample was spread plated on olive oil [2.0 % (v/v)] enriched solid agar medium containing (0.5% w/v $(NH_4)_2SO_4$; 0.05% w/v K_2HPO_4; 0.03% w/v $MgSO_4.7H_2O$ and 1.5% w/v agar, pH 7.0). Colony capable of utilizing the olive as sole source of carbon and energy developed and were isolated. The colonies were individually streaked on modified lipase

assay medium (1.5% w/v peptone; 0.5% w/v NaCl; 0.1% w/v $CaCl_2$ and 1.5% w/v agar) amended with 1% (v/v) Tween 80 at pH 7.2 (Shukla & Gupta, 2007). The plates were incubated for 72 h at 30^{o}C. Colonies surrounded by halozone formed as a result of white precipitate of calcium monostearate was considered as positive for extracellular lipase secretion. Colony with largest halozone was selected and purified on a fresh nutrient agar medium. The purified isolate was characterized using a battery of biochemical tests. Identification to generic level followed the methods of Holt, Krieg, Sneath, Staley & Williams (1994). The isolate was maintained on nutrient agar slant supplemented with 1% olive oil for further studies.

2.3 Preparation of Inoculum

A 48 h old culture of the test organism grown in a medium composed of tryptone 17.0, Soy peptone 3.0, NaCl 5.0, K_2HPO_4 2.5 and was dextrose 2.5 (g/l): without agar on a rotary shaker (150 rpm) at room temperature (28 ± 2^{O}C) and was harvested by centrifugation (6000 rpm, 10 min). Harvested cells were washed twice in sterile deionized distilled water and suspended therein and the optical density (A_{540}) of the cell suspension adjusted to 0.5. This was used as inoculum.

2.4 Medium Composition and Lipase Production

The extracellular lipase production was carried out as described by Gupta, Upadhyay & Shrivastava, (2011). The basal medium (1.0% w/v glucose, 1.0% w/v K_2HPO_4, 5.0% w/v $(NH_4)_2SO_4$, 0.3% w/v $MgSO_4.7H_2O$, 0.1% w/v $CaCl_2$ and 6.0% v/v olive oil, at pH 7.2) was placed in 250 ml Erlenmeyer flasks. The medium was sterilized by autoclaving at 121^{O}C for 15 min after which the flask was inoculated with the test organisms upon cooling and incubated at desired temperature (30^{O}C) in a shaking incubator (120 rpm) for 120 h. At predetermined time, samples were withdrawn and cells were then removed by centrifugation at 7,000 rpm for 15 min. The resulting cell free supernatant as crude lipase preparation was used to assay for lipase activity.

2.5 Lipase Activity Assay

Lipase activity was measured by titrimetric method with 0.05M NaOH using olive oil emulsion as substrate (Burkert, Maugeri & Rodrigues 2004). Briefly, the reaction mixture composed of 5 ml of olive oil emulsion (25 ml of olive oil and 75 ml of 7% Arabic gum solution) in 2 ml of 10 mM phosphate buffer (pH 7.0) was homogenized in a blender for 5 min. Thereafter, the enzymatic reaction started by adding 1 ml of the culture supernatant. The assay was carried out at 37^{O}C for 30 min with orbital shaking incubator. After the time interval, the reaction was immediately stopped by addition of 15 ml of acetone–ethanol mixture (1:1 v/v) and the amount of liberated free fatty acids were titrated with 0.05 N NaOH using thymolphthalein indicator. The amount of NaOH required to reach endpoint (colourless to blue) was recorded. The reaction mixture without the enzyme was titrated in the same way and used as blank. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of fatty acids equivalent per minute under assay conditions (Gombert, Pinto, Castillo & Freire, 1999).

$$. Lipase\ activity\ (\mu mol/\min) = \frac{Volume\ of\ alkali\ consumed \times normality\ of\ alkali}{Time\ of\ incubation \times volume\ of\ enzyme\ solution}$$

2.6 Effects of Lipidic Substrates

The effect of lipid substrates on the biosynthesis of lipase by the test organism was carried out as described by Gupta *et al.,* (2012) with little modification. The medium for the assay consist of the following: S: 0.5% w/v $(NH_4)_2SO_4$, 0.05% w/v K_2HPO_4, 0.03% w/v $MgSO_4.7H_2O$ and olive oil substituted by different lipidic compounds (1% v/v) such as coconut oil cake, palm kernel oil, groundnut oil cake and Tween 80 respectively. The sterile medium contained in 250 ml Erlenmeyer flasks was then inoculated with 2 ml of the test organism after which the flasks were incubated (37^OC) on a rotary shaker (150 rpm) for 72 h. Thereafter, cells were removed by centrifugation at 7,000 rpm for 20 min and cell free supernatant obtained was used directly as crude enzyme to determine the lipase activity as described earlier.

2.7 Effect of Nitrogen Sources

The ammonium sulphate ($(NH_4)_2SO_4$) contained in the basal lipase production medium was replaced with 1% w/v of different nitrogen sources such as urea, yeast extract, ammonium chloride and potassium nitrate respectively. The medium was sterilized by autoclaving at 121^OC for 15 min and thereafter allowed to cool at room temperature. This was followed by inoculating the medium with 2.0 ml of the test organism and then was incubated on a rotary shaker for 72 h. The pH and incubation temperature was maintained at 7.2 and 37^OC respectively. The supernatant obtained after the incubation was used to determine the lipase activity as described earlier.

2.8 Effect of pH

The effect of pH on the extracellular production of lipase in the culture medium was determined in the pH value range from 5.5 - 9.5 was assessed as described by Kumar & Valsa (2007). The pH of the culture medium was adjusted with phosphate buffer solution. The medium was sterilized at 121^OC for 15 min after which it was inoculated with the test isolate and incubated on a rotary shaker (150 rpm) for 72 h. The supernatant obtained after incubation was used to determine the lipase activity.

2.9 Effect of Incubation Time

The effect of incubation time on the lipase production by the test organism was adopted as described by Hasan & Hameed (2001). The assay was carried out for 96 h using different carbon sources. Samples were collected after every 24 h of incubation to determine for the production of lipase using the cell free supernatant. Lipase activity was estimated as described earlier.

2.10 Effect of Inoculum Size

The effect of cell inoculum size on the lipase production was determined as described by (Tehreema, Mubashir, Rukhsana & Ikram, 2011) with little modification. Briefly, lipase

production medium was inoculated respectively with 2.0 ml of 0.1, 0.2, 0.4, 0.6 and 0.8 optical density (OD) spectrophotometrically standardized inoculum of the test organism at absorbance of 540 nm (A_{540nm}). The medium was incubated at 37^{O}C for 72 h and the crude lipase preparation from these cultures was used to estimate the lipase activity by titrimetric method as described earlier.

3. Results and discussion

The organism used for the study was identified as *Bacillus* sp. *Bacillus species* are Gram positive bacteria that are nutritionally versatile. They have been attracting growing interest in industrial applications because they are known to be involved in the production of a number of economically valuable products (Aravindan, Anbumathi & Viruthagiri, 2007; Imamura & Kitaura, 2000). Being one of the predominantly soil resident organisms, *Bacillus species* lend itself amenable for use in the industrial production of lipases.

Lipase production profile of the test organism is as shown in Table 1. The results obtained indicated that enzyme production increased with increasing time and maximum enzyme activity (20.17 ± 3.6 µmol/min) was obtained after 72 h of incubation and thereafter there was progressive decrease in activity as time increases. Prasad (2013) in his work reported that the highest lipase activity (7.20 µmol/min) was obtained at 45 h of incubation (30^{0}C) when *Serratia marcescens* isolated from palm oil mill effluent samples employed to assess the optimization of culture conditions for lipase production. The differences in highest lipase activity may be as a result of type of bacterial strain employed. The lowest lipase activity with a value of 9.78 ± 1.1µmol/min was obtained at 24 h of incubation.

Table 1: Lipase Production Profile of *Bacillus* sp. PME

Time (h)	Lipase activity (µmol/min)
24	9.78 ± 1.1
48	12.07 ± 2.8
72	20.17 ± 4.6
96	17.11 ± 3.1
120	14.56 ± 2.3

Within the lipidic substrates (carbon sources), the lipase activity ranges from 20.78 ± 0.1 to 22.78 ± 0.3 µmol/ml. Among the selected substrates, the highest and lowest lipase activity was obtained in palm kernel oil and coconut oil cake with values of 22.78 ± 0.3 and 20.78 ± 0.1 µmol/ml respectively after 72 h of incubation (Table 2). The present study is in contrast with the findings of Mohan, Palavesam & Ajitha, (2012) who reported that the production of lipase was high in medium containing coconut oil sediment with *Vibro* species isolated from a fish gut. Lipids such as those used in this work are found to be an inducer of lipase production and this was observed in lipase production by *Bacillus coagulans* and *Vibro*

species (Mohan *et al,* 2012; Kumar & Valsa 2007). Gayathri *et al.,* 2013 reported that oil from other natural sources such Sunflower oil, soybean oil, coconut oil as well as palm oil can influence the expression of lipase activity in many of microbial strains.

Table 2: Effect of Different Lipidic Substrates on Lipase Biosynthesis by *Bacillus* sp. PME

Substrate	Lipase activity (µmol/min)
Coconut oil cake	20.78 ± 0.3
Palm kernel oil	22.78 ± 0.1
Groundnut oil cake	21.17 ± 0.2
Tween 80	21.11 ± 0.4

Figure 1 depicted the effect of various nitrogen sources on lipase production by the test isolate. In order to study the effect of nitrogen sources some organic and inorganic nitrogen sources were used. Addition of yeast extract resulted in maximum lipolytic activity (6.8 ± 0.4 µmol/min) followed by urea (6.2 ± 0.2 µmol/min). Similar results was obtained by Mukesh-Kumar & Valsa (2012) who assessed the production, optimization as well as the purification of lipase from *Bacillus* sp. MPTK912 isolated from oil mill effluent. It has observed that among the various nitrogen sources employed in assessment of lipase production by microbial strains, organic nitrogen sources have been yielding maximum lipase activity than inorganic nitrogen sources (Sirisha *et al.,* 2010).

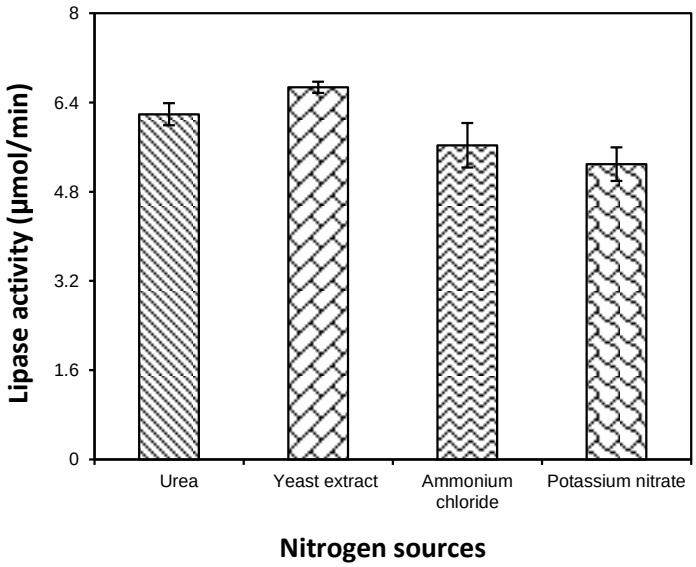

Figure 1: Effect of nitrogen sources on lipase production by *Bacillus* sp. PME.

The results obtained from the effect of pH on lipase activity of the test isolate are shown in figure 2. Result shows that highest lipase activity for the test isolate was achieved at pH value of 7.5 at 37^0C after incubation period of 72 h with lipase activity value of 6.7 ± 0.1 µmol/min. The lowest lipase activity was observed in pH value of 5.5 with lipase activity of 4.8 ± 0.1 µmol/min. This may be as a result of changes in the optimum external pH which may alter the ionization of nutrient molecules of the medium hence reduced their availability to the organism (Tehreema *et al.,* 2011). The highest lipase activity obtained in this work at pH of 7.5 is similar to the result obtained by Prasad (2013) in his work at pH of 7.0 in his assessment of production of extracellular lipase by *Serratia marcescens* isolated from industrial effluent. Gupta, *et al*., (2004) in their review work reported that maximum activity of lipases produced by bacterial strains is observed at pH values above 7.

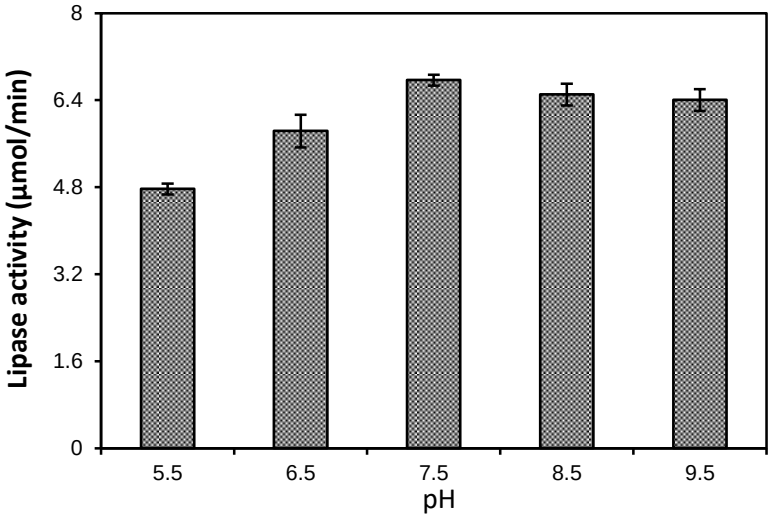

Figure 2: Effect of pH on lipase production by *Bacillus* sp. PME

Figure 3 shows the influence of incubation time on the lipase production by test organism. There was progressive increase in lipase activity as incubation time increases in all the different lipidic substrates supplemented media. The highest lipase activity was obtained after 72 h of incubation in the presence of all the lipidic substrates with values of 21.8 ± 0.4, 18.9 ± 0.2, 19.6 ± 0.3 and 21.1 ± 0.4 µmol/min respectively and thereafter lipase activity started steady decrease. The fall in enzyme activity may be as a result of exhaustion of nutrients in substrate which resulted in the inactivation of enzyme (Tehreema *et al.,* 2011). Kais, Ahmed and Maahir, (2014) in their study reported that it might be due to the .absorption of the enzyme by the substrate or by the proteolytic activity. The result obtained with the test isolate is in corroboration with the results obtained by Mukesh-Kumar, Rejitha, Devika, Balakumaran, Immaculate & Kalaichelvan (2012); and Hasan & Hameed (2001) in their various works as they found that lipase production by *Bacillus* sp. MPTK 912 isolated from oil mill effluent and *Bacillus species* isolated from soil and wastes of tanneries respectively were maximum at 72 h.

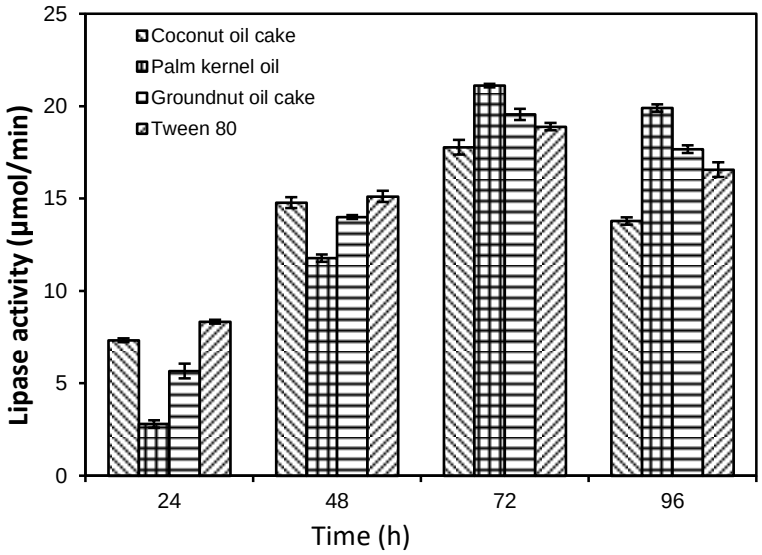

Figure 3: Effect of incubation time on lipase production by *Bacillus* sp. PME hydrolyzing various lipidic substances.

The results on the effect of inoculum size are as depicted in Table 3. The results revealed that the lipase production was decreased with increase in cell number of the test organism and optimal inoculum size was found to be at cell optical density of 0.2 with 15.3 ± 0.4 µmol/min lipase activities. This was followed by lipase activity of 12.3 ± 0.3 µmol/min obtained at cell optical density of 0.1. The least lipase activity of 9.7 ± 0.2 µmol/min was obtained at cell optical density of 0.8. The gradual decline in enzyme activities observed with higher inoculum levels (0.4 to 0.8 cell optical density) may be probably attributed to the insufficiency of some nutrients owing to fast bacterial growth as a result of high number cells (Niyonzima and More, 2014).

Table 3: Effect of Inoculum Size on the Production of by the Test Isolate

Inoculum size (A_{540nm})	Lipase activity (µmol/min)
0.1	12.3 ± 0.3
0.2	15.3 ± 0.1
0.4	12.2 ± 0.2
0.6	11.8 ± 0.2
0.8	9.7 ± 0.4

Although, Deyaa, Fotouh, Bayoumi and Hassan (2016) in their study reported that various bacterial strains have different optimal inoculum size (cell density) for maximum production of extracellular lipase enzymes in a medium. Therefore, the effect of inoculum size on enzyme production may depend on the bacterial efficiency, size, type and stability.

In conclusion, the study revealed that controlling the cultural conditions and modifying the composition of the medium greatly influences lipase enzyme production and that its

optimization needs a careful manipulation of the cultural environment. From the findings it may be suggested that purified *Bacillus* sp. from the palm oil mill effluent can be employed as a potential industrial microbe in the future.

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