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# PRODUCTION OF LIPASES IN SOLID-STATE FERMENTATION BY *Aspergillus niger F7-02* WITH AGRICULTURAL RESIDUES

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**ABSTRACT**

In this study mould strains screened and molecularly identified as *Aspergillus niger* F7-02 was used to produced extracellular lipase in Solid State Fermentation (SSF) process. Different agricultural residues were combined in different ratios as carbon, nitrogen and elemental sources in the solid culture medium. The optimization of the culture medium was carried out for such parameters as incubation time (24 h - 96 h), inoculum concentration (0.5 – 3.0%, w/v), initial moisture content (40 – 70%, w/v), and initial pH (6 – 8) for maximum yield. The maximum lipase activity of 76.7 U/ml was obtained with a medium containing rice bran (RB), palm kernel cake (PKC), groundnut cake (GNC) and starch (S) at the ratio of 5:5:3:1 (%w/w) with optimum conditions of 60% moisture, 1% inoculum and a pH of 7.0 with an incubation temperature of 30 °C and incubation time of 72 h.

**Keywords:** Agricultural waste, *Aspergillus niger F7-02*, lipase, optimization, solid state fermentation

## INTRODUCTION

Lipases are hydrolytic enzymes that act in aqueous-organic interfaces, catalyzing the cleavage of ester bonds in triglycerides and producing glycerol and free fatty acids (**Freire and Castilho, 2000**). There has been a growing interest for lipases of microbial origin since the eighties. Lipases find an increasing range of applications due to the different catalytic reactions and their regio and enantio selectivity in detergents, foods, pharmaceuticals, fine chemicals, leathers and pulp and paper industries **(Freire and Castilho, 2000**). However, the development of low-cost processes for the production of lipases create a greater industrial application for these enzymes. In this context, solid-state fermentation (SSF) is an interesting low-cost alternative for the production of biomolecules. Solid State Fermentation has great potentials due to its simplicity of operation, low capital cost and high volume productivity **(Akpan *et al.*, 1999)** and has gained renewed interest because of its potential to produce higher yields of fungal metabolites than submerged fermentation **(Akpan and Adelaja 2004).** In SSF, agroindustrial residues can be employed as culture medium. These low-cost and abundant raw materials contribute to reduce production costs **(Freire and Castilho, 2000)**. Many agricultural products from cereals or legumes are cheap and readily available in the developing countries as sources of carbohydrates and proteins, so they could provide the required nutrients in the fermentation medium **(Akpan *et al.* 1999)**. The use of agricultural products such as wheat bran solid medium for enzyme production has been well established, but it is scarce in the tropics. However, alternatives such as rice bran solid medium require supplements such as yeast extract and peptone; but these are expensive materials in the tropics **(Akpan *et al.* 1999).** The objective of our study was to investigate the synergistic effect of various agricultural residues (rice bran, palm kernel cake, soy bean, groundnut cake and starch) combined in different ratios (as sources of carbon, nitrogen and elemental supplements) as solid media to support the growth of *Aspergillus niger* F7-02 for lipase production.

## MATERIALS AND METHODS

### Source of microorganisms

Mould strains were isolated from soil samples obtained from a compost farm at the Federal University of Agriculture, Abeokuta, Nigeria. Screening was done by plating decimal dilutions (0.1ml) of suitably diluted soil samples on Sabourand dextrose agar (SDA) incubated at 30°C for 48 h. Pure isolates were obtained by sub-culturing and were maintained on SDA agar slants at 4 °C and sub-cultured bimonthly.

### Chemicals

Tween 80 and Bromocresol green dye (3',3',5',5" Tetrabromo-m-cresolfonephthalein) were purchased from SIGMA CHEMICALS LTD, USA., PCR ladder, Ethidium bromide, PCR Master mix (NORGEN), Primers ITS 4 - Reverse (MACROGEN INC. USA) and ITS 86- Forward (EUROFINS MWG OPERON, HUNTSVILLE) TEXAS, UNITED STATE. All chemicals were of analytical grade (ANALAR).

### Substrates

Rice bran (RB), Palm kernel cake (PKC), Soy bean (SB), Groundnut cake (GNC) and Starch(S) were purchased from KUTO market, ABEOKUTA SOUTH LOCAL GOVERNMENT AREA, OGUN STATE, NIGERIA.

### Screening of lipase positive moulds

This was carried out according to the method described by **Akpan (2004).** The plate medium consisted of Sabouraud dextrose agar (SDA) and 0.1% bromocresol green. The medium was sterilized at 121 °C for 15 min. While still warm, sterile Tween 80 (1%) was added and mixed properly. Sterile plates were poured and inoculated with pure isolates of *A. niger* F7-02 and were incubated at 30 °C for 72 h. Lipase production was detected by a colour change (green to yellow) around the colonies. The diameter of the zones of hydrolysis were measured with a Venier caliper. Mould strains with the widest zone of hydrolysis were picked and stored at 4 °C for further studies.

### Identification of lipase positive moulds

The lipase positive moulds were identified according to the methods of **Barnett and Hunter (1972)**. Wet preparation of isolates were prepared with Lactophenol blue and observed under X40 power objective lens of a light microscope.

Nucleotide sequences were determined by analysis of fluorescently labeled DNA products generated by 2X PCR Master mix on an AB373a Strech (short gun) DNA sequencer. Reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and forward primer ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') were used in all sequencing reactions. The obtained sequences of genomic DNA were aligned by submitting them to the non-reductant nucleotide database at Genbank using the BLAST program in order to determine the identity of the isolates (http://www.ncbi.nlm.nch.gov).

**Preparation of inocula**

Fungal spores (1 g) were suspended in 10 ml of sterile water and 1 ml (10% w/v) of suspension was used as inocula **(Kareem and Akpan, 2003)**.

**Media development**

Six different solid fermentation media were formulated using various agricultural residues to support the growth of the moulds for lipase production: rice bran (RB), palm kernel cake (PKC), soy bean (SB), groundnut cake (GNC) and starch (S) combined in following ratios:

Medium I: RB, PKC, GNC and S (5:5:3:1 %w/w);
Medium II: RB, GNC, S (10:3:1 %w/w);
Medium III: PKC, GNC, S (10:3:1 %w/w);
Medium IV: RB, SB, S (10:3:1 %w/w);
Medium V: RB, SB, S, V1 (10:3:1 %w/w) + Vegetable oil (1 ml);
Medium VI: RB, SB, S,V2 (10:3:1 %w/w) + Vegetable oil (2 ml);
Each medium was sterilized at 121 $^{o}$C for 15 min and inoculated with 1 ml of spore suspension of *A.niger* F7-02 and incubated at 30 $^{o}$C for 72 h. Various process parameters such as incubation time (24 – 96 h); inoculum concentration (0.5 – 3.0% w/v); solid substrate moisture content (40 - 70%) and initial pH (6.0 - 8.0) were optimized by conventional method for maximal lipase production and their effects monitored.

**Crude enzyme recovery from mouldy rice bran**

This was carried out using the method of **Kareem and Akpan (2003).** Mouldy rice bran was dissolved in 50 mM sodium phosphate buffer pH 8.0 and the mixture was incubated at 4 $^{o}$C for 3 h with intermittent shaking. The filtered extract was used as crude enzyme source.

**Titrimetric assay of lipase activities**

This was carried out by combined methods of **Praphan and Kirk (2001)** and **Janaina *et al.* (2006)**. Lipase activities were assayed with olive oil emulsion substrate which was prepared by mixing 25 ml of olive oil and 75 ml of 7% Arabic gum solution in a rotary shaker at 150 rpm for 5 min. The reaction mixture contained 50 ml of olive oil emulsion substrate and 10 ml of crude enzyme which was incubated at 50 ºC for 30 min under orbital shaker at 160 rpm. At five suitable reaction intervals (5, 10, 15, 20, and 25 min), 5 ml reaction mixture was removed and each subsample was transferred to a separate flask containing 10 ml of 95% (v/v) ethanol and three drops of 1% (w/v) thymolphtalein indicator and was swirled to stop the reaction. The amount of released free fatty acids was titrated with 0.05 N NaOH solutions and calculated according to the following equation.

$$\mu mol\ fatty\ acid(ml\ subsample) = \frac{[(ml\ NaOH\ for\ sample - ml\ NaOH\ for\ blank) * N * 1000]}{5\ ml}$$

Where *N* is the normality of the NaOH titrant used (0.05 in this case)

One unit (U) of lipase activity is the amount of enzyme which liberates from emulsion substrate 1 µmol of fatty acid per ml per minute under specific assay conditions.

**RESULTS**

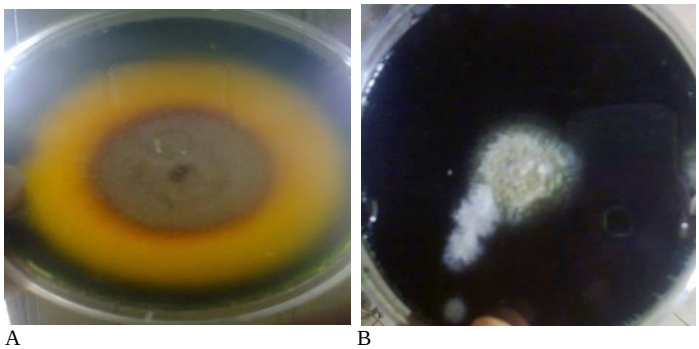
**Isolation and screening of lipase positive moulds**

Of the fifty moulds isolated from the soil samples only five were positive for lipase production and only one was selected because of its widest zone of hydrolysis and high lipase activity as shown in Table 1. Appearance of zone of hydrolysis around *A. niger* F7-02 (Plate 1a) showed that the isolate was lipase positive while (Plate 1b) showed a non-lipolytic mould. Hydrolysis of olive oil by lipase producing moulds led to the release of fatty acid and glycerol and colour change in the medium from green to yellow due to the chromogenic bromocresol green indicator and change in the pH of the medium

**Table 1** Screening of microorganisms for lipase production

| Microorganisms | Clearance zones (mm) | Lipase activity (U/ml) |
|---|---|---|
| *Aspergillus niger F7-02* | 7.0 | 70.6 |
| *Rhizopus sp* | 5.1 | 60.9 |
| *Penicillum sp.* | 4.0 | 41.7 |
| *Aspergillus oryzae* | 0.5 | 12.3 |

*Mean of triplicate determinations



A B

**Plate 1** Lipase positive of *Aspergillus niger* F7-02 with clear zone of hydrolysis (A) and lipase negative (B) isolates on Bromocresol green agar

**Molecular identification**

The internal transcribed spacer (ITS) regions of ITS 86 and ITS 4, which are located between the highly conserved small (18S) ribosomal subunit genes in the rRNA of the lipase producing strain have sufficient sequence variability and were used for the identification of the isolate as *Aspergillus niger* F7-02. The molecular identification confirmed the isolate align with *A. niger* F7-02 of assertion number JN561274 with 98% identity matching.

**Optimization studies on Solid State Fermentation Process for lipases production**

All the six media supported the growth of *A. niger* F7-02 with highest lipase activity of 76.7 U/ml after 72 h of incubation in medium 1 containing RB, PKC, GNC, S (5:5:3:1 %w/w) (Figure 1). Medium II which contained RB, GNC, S (10:3:1 %w/w) had a lower activity of 38.5 U/ml when compared to medium III which consisted of PKC, GNC, S (10:3:1 %w/w) with activity of 57.4 U/ml, which is the closest to activity recorded in medium I. Like the conventional rice bran solid substrate, medium IV [(RB, SB, S) 10:3:1 %w/w], had an activity of 49.5 U/ml, and when vegetable oil was added (medium V) [(RB, SB, S, V1) 10:3:1:1] to see the inducing effect, the activity increased to 55.5 U/ml. However, in medium VI, where the concentration of vegetable oil was increased to 2 % w/v ratio, there was a decrease in lipase activity to 48.7 U/ml. This suggested that there was inhibition of enzyme synthesis and this could be attributed to feedback inhibition by the higher lipid concentration of medium VI.

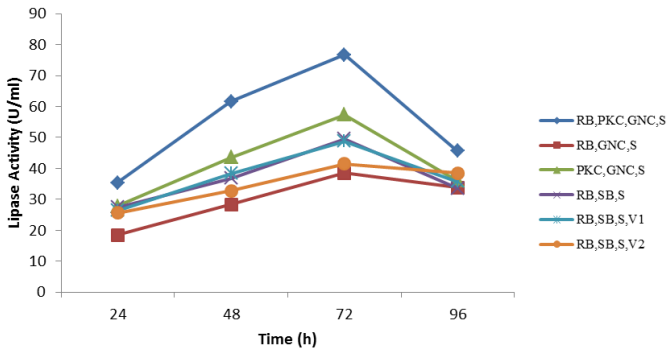



**Figure 1** Lipase activity of *Aspergillus niger* F7-02 from different formulated media

**Factors affecting lipase activities of *A. niger* F7-02**

The effect of incubation time on lipase activities of *A. niger F7-02* can also be seen in Figure 1. The lipase activity of *A. niger F7-02* in medium I increased with incubation time and peaked at 72 h with activity value of 76.7% U/ml. In this study, maximum lipase activity was observed after 72 h of fermentation. At longer incubation periods, lipase activity decreased as nutrient and oxygen supply became growth limiting and also due to accumulation of toxic end products.

The effect of different inocula concentration of *A. niger F7-02* on lipase activity at 72 h incubation time is shown in Figure 2. Inoculum
levels had effect on lipase activities with optimum activity at 75.4 U/ml at 1ml (1% w/v) inoculum concentration. Thus lipase activity increased with inoculum concentration and peaked at 1.0% w/v concentration.
The effect of the initial moisture content of the solid substrates on lipase activity of *A. niger F7-02* is shown in Table 2. At varying moisture percentages with 1.0% inoculums concentration incubated at 30 ºC for 72 h. In this study the maximum lipase activity (76.0 U/ml) was observed at 60% (w/v) moisture content at 76.0 U/ml.

The effect of initial pH medium on the lipase activities of was investigated within pH range of 6.0-8.0, at 60% (w/v) moisture level, 1.0% inoculums level and 72 h incubation period. Lipase activity increased with pH with peaked at 7.0 as shown in Figure 3.

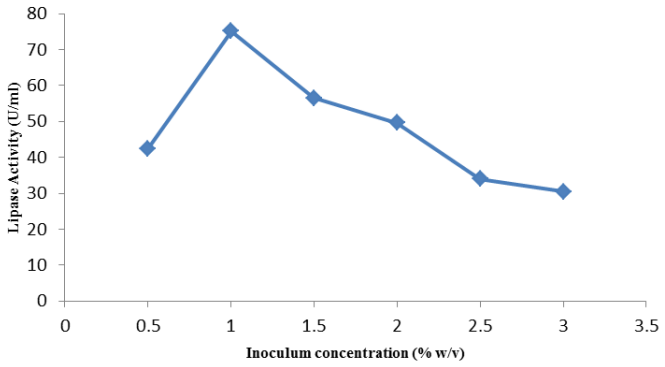



**Figure 2** Effect of inoculum concentration on lipase activity of *A. niger F7-02*

**Table 2** Effect of initial moisture content of the solid substrates on lipase activity of *A. niger* F7-02

| Moisture level (% w/v) | Lipase Activity (U/ml) |
|---|---|
| 40 | 20.3 |
| 45 | 25.0 |
| 50 | 42.2 |
| 55 | 58.5 |
| 60 | 76.0 |
| 65 | 50.7 |
| 70 | 36.5 |

*Mean of triplicate determinations

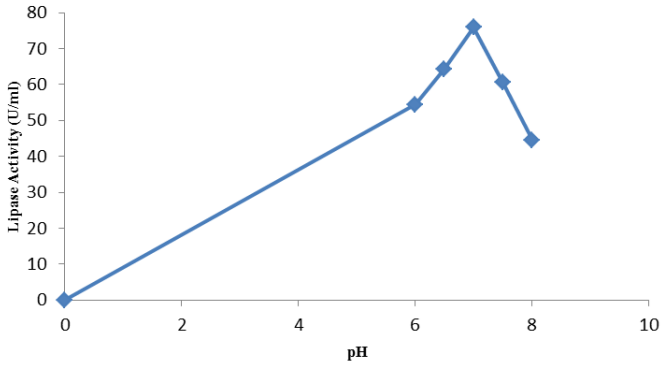



**Figure 3** Effect of medium pH on lipase activitity of *A. niger F7-02*

**DISCUSSION**

**Isolation and screening of lipase positive moulds**

Soil has been identified as the most diverse of terrestrial habitats **(Hunt *et al,* 2004)** and the microbial community plays a fundamental role in decomposition. This prompted the search for lipase producing strains from the soil community in which microorganisms have been labeled as a rich source of new biocatalysts. Hydrolysis of olive oil by lipase produced moulds lead to the release of fatty acids and glycerols while the chromogenic dye ; bromocresol green, gave a characteristic colour change from green to yellow as a result of pH change in the medium. This observation conformed with the findings of **Akpan (2004)**, who also stated that bromocresol agar medium is a simple and inexpensive medium, compared to spirit blue agar or triolein agar, which are conventionally employed for the screening of lipolytic microorganisms **(Magda *et al.*, 2004)**.

**Molecular identification**

Use of traditional methods for classification and identification of fungi has been reported to have certain drawbacks **(Deene and Lingappa, 2012)** such as its non-applicability for non-cultivable organisms and also occasionally, biochemical characteristics of some organisms do not fit into patterns of any known genus and species. Molecular techniques which involves the amplification and sequencing of target regions within the ribosomal DNA gene has been reported by **Buzina *et al.*, (2001); Iwen *et al.*, (2002); Rakeman *et al.*, (2005); Schwarz *et al.*, (2006)** as a useful adjunctive tool for the identification of fungi. The internal transcribed spacer (ITS) regions of ITS 86 and ITS 4, which are located between the highly conserved small (18S) ribosomal subunit genes in the rRNA of the lipase producing strain have sufficiently sequence variability. This corresponds to the findings of some researchers **(Brandt *et al,* 2005; Deen and Lingappa, 2012)** who had successfully used the ITS regions for fungi identification to species level.

**Optimization studies on Solid State Fermentation Process for lipases production**

The use of agricultural residues which is renewable and abundantly available as substrates for lipases production has been reported. Babassu oil cake **(Gombert *et al*., 1999),** olive cake and sugar cane bagasse **(Cordova *et al*., 1998),** gingelly oil cake **(Kamini *et al*., 1998)**, wheat bran **(Gwen *et al*., 2006)**, rice bran **(Rao *et al*., 1993)**, *Jatropha curcas* seed **(Mahanta *et al*., 2008, Osho, 2013)**, palm kernel cake **(Sarat *et al*., 2010)** and groundnut cake **(Manoj *et al*., 2010),** have all been used for lipase production. In this study, the effect of various agricultural residues (rice bran, palm kernel cake, soy bean, groundnut cake and starch) combined in different ratios as sources of carbon, nitrogen and elemental supplement to support growth of *A. niger* F7-02 for lipase production was observed. **Osho *et al*., (2014)** had earlier reported the use of rice bran-physic nut waste cakes as a solid medium for *Alternaria sp.* MGGP 06 growth by direct incorporation (immobilization) with vegetative sponge for lipase production. This result showed there is synergistic effect in combining medium supplements for high lipase activity when compared with activity of 48.4 U/ml when wheat bran was used only **(Gwen *et al*., 2006),** 18.58 U/ml with PKC only **(Sarat *et al*., 2010)** and 45.7 U/ml with GNC only **(Manoj *et al*., 2010)**. This result clearly showed that PKC has important constituent element required for lipase activity, which is lacking in rice bran. Like the conventional rice bran solid medium of **Akpan and Adelaja (2004).** However, in medium F, where the concentration of vegetable oil was increased to 2 % (w/v) ratio, there was a decrease in lipase activity to 48.7 U/ml suggesting inhibition of enzyme synthesis which could be attributed to feedback inhibition by the higher lipid concentration of medium VI.
Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, dissolved oxygen concentration, inoculums level and incubation time **(Elibol and Ozer, 2001)**. Many researchers have reported different incubation periods for optimal lipase production; maximum lipase activity was achieved after 120 h by **Mahadik *et al*., (2002),** with *Aspergillus niger* on wheat bran, **Sarat *et al*., (2010)** reported maximum lipase activity by *Yarrowia lipolytica* after 96 h incubation on PKC as substrate. In another study, **Benjamin and Pandey (1997)** reported maximum production of lipase by *Candida rugosa* after 72 h incubation.
Reduced activity was observed with lower and higher inoculum levels which may be attributed to insufficient biomass leading to reduced product formation and too much biomass leading to the poor product formation **Sarat *et al*., (2010)**. Many investigators have reported different optimal inoculum levels for lipase production with different microorganisms. For *Rhizopus oligosporus*, 1 ml inoculums was reported by **Ul-Haq *et al*., (2002),** while **Kamini *et al*., (1998)** reported inoculums concentration of $1.07x10^8$ spores/10 g of substrate. **Sarat *et al*., (2010)** used an inoculum concentration of 2 ml (20% w/v) for maximum lipase production by *Yarrowia lipolytica* in PKC substrate.
Initial moisture content of substrate has been reported to play a key role in the microbial growth and for effecting biochemical activities in solid state fermentation **(Babu and Rao, 2007).** In this study, maximum lipase activity was

observed at 60% (w/v) initial moisture content at 76.0 U/ml. **Mahanta *et al.*, (2008)** reported 50% (w/v) initial moisture as ideal for lipase production in *Jatropha curcas* seed cake as substrate, while 70% (w/v) initial moisture content was reported by **Sarat *et al.*, (2010)** with PKC as substrate. Decrease in lipase production due to higher moisture content has been attributed to decrease in porosity and hence decrease in gaseous exchange leading to suboptimal growth of microorganisms **(Silman *et al.*, 1979; Sarat *et al.*, 2010)** while decrease in lipase activity at lower moisture content has been reported by **Perez-Guerra *et al.* (2003)** to be due to the reduction in the solubility of nutrients of the substrate which led to lower degree of swelling and also created higher water tension.

Lipase activity has been reported to be affected by the pH of the medium as pH is an important parameter required for microbial growth **(Sarat *et al.*, 2010)**. The result obtained in this study, showed effect of pH of medium on lipase activity, with optimum activity recorded at pH 7.0. **Manoj *et al.*, (2010)** also reported an optimum activity at pH 8.0 with *Bacillus subtilis* OCR-4 when GNC was used as a substrate.

## CONCLUSION

This study has indicated that combining agricultural residues as sources of carbon, nitrogen and elemental supplements in solid state fermentation to support the growth of *A. niger* F7-02 for lipase production led to higher lipase yield and is cost effective as compared with other studies that used single waste with expensive elemental supplements.

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