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RESEARCH ARTICLE

Lipase Production by Fungal Isolates from Palm Oil-Contaminated Soil in Awka Anambra State, Nigeria

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ABSTRACT

Fungi isolated from oil-contaminated soil around *Eke-Awka* market in Awka, Anambra State, Nigeria, were accessed for lipolytic activity on modified lipase assay medium. *Mucor sp.*, *Aspergillus flavus* and *Candida sp.* showed the highest activity as accessed on colony diameter to calcium monolaurate precipitate ratios. Lipase production was studied in both shake, still fermentation systems and mycelia mass was determined. In still cultures *Mucor sp.* and *Aspergillus flavus* showed greater activity after 120 hours whereas, in shake culture *Candida sp*. showed greater activity. *Mucor sp*. showed a marked increase in activity (40U/ml) after 168hours for the still culture and a reduction in activity for the shake culture (0.60 U/ml). Lipases can be produced with still-culture systems using strains of *Candida sp*. These findings suggest that agitation is an important factor in the production of lipase as it influences biomass production.

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INTRODUCTION

Lipases are glycerol ester hydrolases (E. C. 3.1.1.3), which hydrolyze tri-, di-, and mono-glyceride ester linkages at water-oil interface. They constitute a diverse and ubiquitous family of enzymes that in biological systems initiate the catabolism of fats and oils by hydrolyzing the fatty acylester bonds of acylgycerols (Carriere *et al*., 1994 and Jose *et al*., 2004). During hydrolysis, an acyl group is transferred from glycerides to lipases forming lipase-acyl complex, from which the acyl group is transferred to the -OH group of water.

However, in non-aqueous conditions, these naturally hydrolytic enzymes can acylate alcohols, sugars, thiols and amines, synthesizing a variety of stereo-specific esters, sugar esters, thioesters and amides (Dellamora-Oritz, 1997). In order to efficiently employ a lipase for synthesis, the enzyme must be immobilized since soluble lipases lose their activity in non-aqueous reaction media (Bruno, 2004). These synthetic properties allow wide spread applications in various fields of biochemical and organic conversions (Hsu *et al*., 2002).

It is well known that lipases are the most widely used enzymes in organic synthesis and more than 20% of all biotransformation is performed with lipases (Gitlesen *et al.,* 1997). In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceutical, food and leather industries (Gulati *et al*., 2005; Gunstone, 1999).

Promising fields for the application of lipases also include the biodegradation of plastic and the resolution of racemic mixtures to produce optically-active compounds (Gombert *et al.,* 1999). Some lipases are also able to catalyze esterification, trans-esterification and enantios-elective hydrolysis reactions. These functions of lipases can be attributed to their broad specificity for a wide spectrum of substrates, stability in organic solvents and enantioselectivity (Fadnavis and Deshpande, 2002). Plants, animals and many microorganisms are lipase-producers (Kamimura *et al*., 2001).

Enzyme-producing microorganisms include bacteria (Kulkani and Gadre, 2002), fungi (Fodiloglu and Erkman, 1999), yeast (Corzo and Revah, 1999) and actinomycetes (Sommer *et al*., 1997). Lipases from microorganisms have drawn much attention for their potential use in biotechnology, mainly due to their availability and stability (Ghosh *et al*., 1996).

Several species of bacteria, yeasts and molds produce lipases. These microorganisms produce lipases both by solid-substrate and submerged fermentations. Fungal lipases are derived mainly from *Candida* and *Aspergillus* sp. and *Geotrichum* sp. (Ginalska *et al.,* 2004*).* Earlier

studies on lipase-producing fungi have focused on kinetic parameters, sequencing and cloning of lipase genes, enzyme action, and structural characterization (Espisan *et al.*, 1990 and Rapp, 1995). Typical substrates of lipases are vegetable oil, animal fat, fish oil, olive oil, butter oil, (milk fat), and synthetic TAG such as triolein. Desirable characteristics exhibited by commercially important lipases include: alkali tolerance, and thermostability. Most lipases reported so far are active at neutral pH. In view of the diverse applications of lipases, there has been a renewed interest in identifying new sources for lipases with improved characteristics.

The aim of this work was characterize lipase production by fungi isolated from oil-contaminated around *Eke-Awka* market, Awka, Anambra State, Nigeria. Lipase production was studied in still or shakes fermentation systems and the degree of lipase production was compared amongst different fungal species.

MATERIALS AND METHODS

Isolation of Fungi from soil

Soil samples were collected from oil-contaminated soil in *Eke-Awka* market in Awka, Anambra State. Three grams of soil were collected using a sterile petri-dish, transported to the laboratory and processed immediately. The fungal species present in the sample were isolated using soil dilution method (Booth, 1971) and the cultures were maintained on Sabouraud Dextrose Agar (SDA). 6.5g of medium was dissolved in 100ml of water and autoclaved at 121°C for 15minutes. Fungal species producing lipases were isolated from soil samples by using a selective medium in which olive oil was the source of carbon and energy.

Preliminary screening for lipase production

The isolates growing on SDA were screened for lipolytic activity by point-inoculation on modified lipase assay medium (Sierra, 1957), containing (g/L): peptone, 10; NaCl, 5; Calcium chloride, 0.1;olive oil, 1ml; agar, 20 and distilled water, 1000ml. Plates were incubated at room temperature for 7days.

Lipolysis was indicated by the appearance of white opaque zones around the colonies. The white opaque zones were due to the deposition of calcium monolaurate precipitate. The colony diameter of the isolates and that of the calcium monolaurate were measured and the ratio of the diameters was calculated.

Identification of Fungal Isolates

Pure cultures of the six isolates that indicated lipolysis were grown on SDA plates. The medium was prepared as described earlier. The colony morphology and microscopic characteristics of the isolates were observed. Morphology and microscopy was compared with results in Colour Atlas of fungi (Frey *et al*, 1979, Watanabe, 2002, Ellis *et al*., 2007).

Lipase Production

Production of the enzyme was carried out in shake and still flask cultures for the top-three lipase producers from the preliminary screening. Erlenmeyer flasks (250ml) each containing 60ml of the fermentation medium were inoculated with two loopfuls of isolate K1, K2 and K3. The medium had the following composition (g/L): yeast extract, 5; peptone, 1.5; NaCl, 1; magnesium sulphate.heptahydrate, 1; potassium dihydrogen phosphate, 3; olive oil, 10ml; distilled water, 1000ml, pH 5.5. Fermentation was carried out at 32°C in a rotary incubator at 140 rpm for the shake culture while the still cultures were left undisturbed at room temperature. Lipase activity was measured for the 5 days and 7 days time points and biomass was measured after only for the 7days time points.

Preparation of Olive Oil Emulsion

The olive oil emulsion used for the assay was prepared according to Rua *et al*., (1993). A 10% (w/v) gum arabic solution in water was first prepared. The olive oil emulsion was made by mixing 25% (v/v) olive oil and 75% (v/v) of the 10% (w/v) gum Arabic solution in water. The emulsion was mixed completely and stored at 4°C for not more than two weeks.

Lipase Assay

Lipase activity in the broth or medium was determined titrimetrically based on olive oil hydrolysis (Macedo *et al*., 1997); a modification of the method described by Musantra, (1992). The enzyme was partially purified. The cell-free supernatant was obtained by centrifuging at 5,000rpm for 15minutes. The reaction mixture was composed of 1ml of 0.02M phosphate buffer (pH 7), 0.5ml of 0.03M calcium chloride solution, 0.5ml of 0.03M calcium chloride solution, 0.5ml of olive oil emulsion and 0.5ml of enzyme solution. The mixture was incubated at 40°C for 40 minutes and the reaction was stopped by the addition of 10ml of acetone: ethanol (1:1) mixture. The liberated fatty acids were titrated with 0.1N NaOH solution using 5% (w/v) phenolphthalein as indicator.

A blank was prepared by incubating the reagent mixture of the above composition without the enzyme solution at 40°C for 40 minutes. The reaction was stopped by addition of 10ml of acetone: ethanol (1:1) mixture and titrated as described earlier. The difference between the titre of the blank and that of the reaction mixture gives the amount of alkali required to neutralize the liberated fatty acids expressed as oleic acid. One unit (U) of the lipase activity is defined as the amount of enzyme capable of releasing one milligram of oleic acid per minute under the conditions described above.

Dry Cell Weight Determination

The mycelium from each flask was filtered, washed with sterile water and dried at 110 °C for one hour on filter paper. The mass was determined using Adams automatic balance.

RESULTS

Preliminary Screening for Lipolytic Activity

Of the 15 isolates screened for lipolysis, only 6 isolate displayed lipase production, (Table 1). This was marked by deposition of calcium monolaurate precipitates around the colonies. Based on the measurement of colony diameters (Table 1) isolates K1, K2 and K3 recorded the

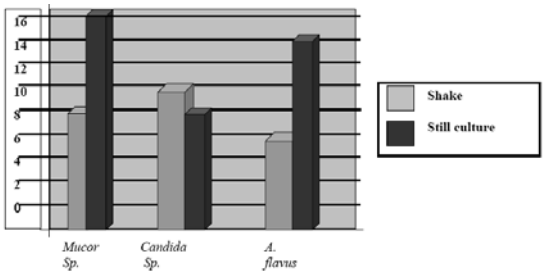

Fig. 1: Lipase activity profile of fungal isolates in 5-day still and shake flask

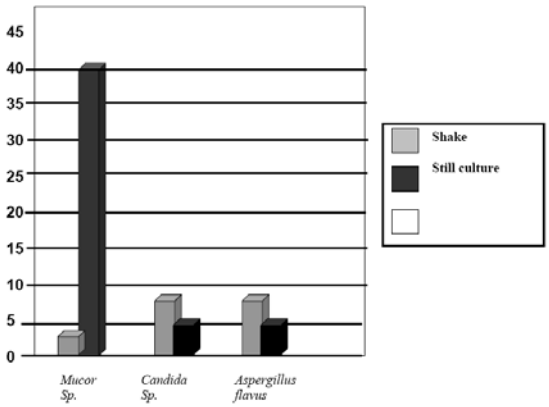

Fig. 2: Lipase activity profile of fungal isolates in 7-day still and shake flask fermentation systems. On the 7th day, the activity chart took a slightly different course.

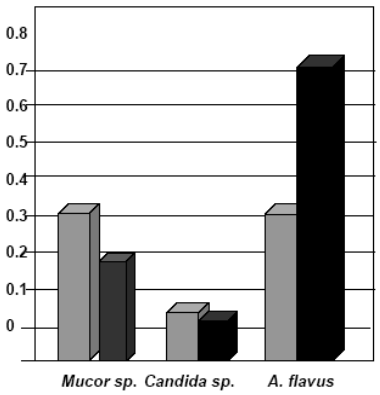

Fig. 3: Mycelial Dry weight production of 7-day still and shake flask fermentation systems.

highest lipase activity with ratios 1.2, 1.1 and 1.1 respectively. Therefore, these three isolates were selected for lipase production in shake and still systems.

Identification of Lipase-producing Isolates

Based on colony morphology and microscopy, the six isolates were assessed as belonging to the genera: *Aspergillus* (four isolates), one isolate each of *Candida* spand *Mucor* sp. Table 2.

Lipase Production

The lipolytic activity of the enzyme was measured after the 5th and the 7th days. The activity profile for both shake and still culture systems were compared (Figs. 1 and 2).

Table 1: Screening of fungal isolates for lipase production

Isolate	Colony diameter (cm)	Calcium-monolaurate diameter (cm)	Ratio
K1	6.5	7.5	1.2
K2	7.8	8.5	1.1
K3	5.8	6.2	1.1
K4	4.3	4.3	1.0
K5	5.8	5.8	1.0
K6	5.3	5.5	1.0

The still culture showed greater activity (14.7 U/ml) for *Mucor sp* and *Aspergillus flavus* than the shake culture. In *Candida sp.* however, the activity was slightly higher for the shake culture (9.5 U/ml). By the 7th day, the activity chart took a slightly different course.

The enzyme activity increased greatly for the still culture for *Mucor sp* (40 U/ml) whereas that of the shake culture declined (0.6 U/ml). *Candida sp* lipase activity also decreased to 6.2 U/ml and 3.4 U/ml for the shake and still cultures respectively. *Aspergillus flavus* did not show any change as activity was constant at 6.2 U/ml for the shake culture. There was however a decrease in activity for the still culture.

DISCUSSION

The best lipase producing fungal isolates namely *Mucor sp.*, *Candida sp*. and *Aspergillus flavus* identified from the plate screening technique, gave consistent activity in submerged culture after 120 hours. *Mucor sp.* gave the greatest cumulative activity (23.7 U/ml) during this period. The fungus was however sensitive to agitation as the still culture produced more extra-cellular lipase. It is likely that agitation, which occurs at 32^0C, denatures the enzyme in the shake culture thus implying low thermostability for lipase produced by the *Mucor* sp. Lipase. This hypothesis is further confirmed by the activity after 168 hours for both culture systems.

Candida sp. showed no variation in its profiles as the enzyme activity was higher for the shake system. The possibility is that *Candida sp.* lipase has a higher thermostability and pH tolerance than that of *Mucor sp.*

Aspergillus flavus did not produce lipase after the 5^{th} day as the reduction in activity was negligible for the shake flask whereas, it is-11.28U/ml for the still culture.

This may be due to massive denaturation of the enzyme within the fermentation vessel.

According to Kamimura *et al.* (1999), biomass production is indicative of intracellular or cell-bound lipase activity. *A. flavus* showed the highest mycelial activity (0.71 g) in still culture indicating high intracellular activity. High intracellular activity implies lower extracellular activity i.e. little secretion of lipase into the medium. This is in line with the activity stated in the profile. *Candida sp.* showed little difference in intracellular activity. *Mucor sp*. gave a pattern consistent with the trend in *A. flavus.*

Conclusion

Screening soil samples for lipolytic molds is a novel and interesting research area with lots of potential towards studying the biodiversity of soil mycoflora. Environmental factors such as pH, temperature and relative humidity play a major role during enzyme

Table 2: Identification of Lipase-producing Isolates

Isolates	Colony morphology	Microscopy	Identity
K1	On SDA, colonies were floccose (cottony in texture), greyish-brown. Growth rate was rapid, thus, colonies filled the entire petri-dish in 3 days. Colour on the reverse side was yellow. Colonies were incubated at 30^{o}C for 5 days.	Sporangiophores were hyaline, erect, non-septate and branched sympodially and circinate. Sporangia were terminal, dark-brown, finely echinulate to smooth and spherical (20- 80 µm in diameter). Sporangiospores were hyaline or pale-brown. Collumellae were ellipsoidal and 4.5-7 x3.5-5 µm in size. Chlamydospores were absent.	*Mucor miehei*
K2	Colonies on SDA were white to cream-coloured smooth and glaborous in texture.	Cells were ellipsoidal to elongate budding blastoconidia, 6-10 x 2-3.5 µm. Sometimes short pseudohyphae may be produced.	*Candida rugosa*
K3	On SDA, colonies were powdery, flat with radial grooves, yellow at first, but later turned to bright to dark yellow-green with age. Colour on the reverse side was creamy. Colonies were incubated at 30^{o}C for 5 days.	Conidial heads were radiate, splitting to form loose columns (300- 400 µm in diameter). Thus, conidiophores stipes were hyaline and coarsely roughened, noticeable near the vesicle and non-septate. Conidia were globose to subglose (3-6 µm in diameter), pale-green and conspicuously echinulate.	*Aspergillus flavus*
K4	On SDA, colonies had rapid growth rate. However, colonies were flat and compact with yellow basal felt covered by a dense layer of black conidial heads with powdery texture. The colour on the reverse side was pale yellow. Colonies were incubated at 30^{o}C for 5 days.	Conidiophores were hyaline or pale-brown, erect, simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial heads (up to 3 mm by 15 to 20 µm in diameter), split into over 4 loose conidial columns with over 4 fragments apically composed of catenulate conidia (over 15 conidia/ chain) borne on uniseriate and biseriate phialides on pale-brown, globose vesicles and phialides acutely tapered at apex. Conidia (3.5-5 µm in diameter) are phialosporous, brown, black in mass globose and minutely echinulate	*Aspergillus niger*
K5	On SDA, colonies were powdery, flat, white at first, then turned yellowish-brown with age. Colour on the reverse side was yellow. Colonies were incubated at 30^{o}C for 5 days	Conidiophores appeared blue and terminated in a vesicle of uniseriate phialides. Conidia were one-celled, rough-walled and produced in long chains which were divergent.	*Aspergillus sp*
K6	On SDA, colonies showed typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores. Texture was powdery and the colour on the reverse side was yellow	Conidial heads were typically columnar (up to 400 x 50 µm and uniseriate. Conidiophore stipes are short, smooth-walled and had conical-shaped terminal vesicles supporting a single row of phialides on the upper two thirds of the vesicle. Conidia were produced in basipetal succession forming long chains and are globose to subglobose (2.5-3.0 µm in diameter), green and rough-walled to echinulate.	*Aspergillus fumigatus*

production and metabolic activities. These studies have the objective of assessing fungal isolates, which can be helpful in degrading lipid wastes from oil mills and other lipid-containing wastes.

Still fermentation systems may soon be the system of choice for lipase producers on the industrial scale, as it has proven very productive. It would save power costs in running agitator shafts and in maintaining heating systems. Moreover, it would prove to be the most promising system for the production of thermo-variable lipases from *Mucor sp.* Further studies are needed to show the growth curve of the organism under this condition.

Candida rugosa lipase could still be produced with agitation, as it showed no sensitivity to it. Additionally, the high biomass production by *A. flavus* could be of advantage in the detergent industry as biomass production started after 24hours incubation.

Isolation and bio-prospecting for lipolytic molds with lipase production ability has a promising future because lipase-based processing is fundamental in modern biotechnology.

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