

SCREENING, OPTIMIZATION AND CHARACTERIZATION OF EXTRACELLULAR LIPASE OF *Aspergillus niger* ATCC 1015

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ABSTRACT

This study focused on screening, production and characterization of strains of microorganisms isolated from groundnut cake wastes capable of producing lipolytic enzyme. Over one hundred isolates were screened on Bromocresol green medium to detect the presence of lipase producing organisms by a colour change of the medium from green to yellow around the colonies at pH 3.8 – 5.6. Two of the isolates with NCBI Accession number ACJE01000015.1 and NT-166520.1 were identified as *Aspergillus niger* ATCC 1015 and *A.niger* CBS 513.88 respectively based on the nucleotide sequence of the domain of DNA gene. Other lipase producers include *A. niger* (B-05, B-17, B-33), *A. oryzae* (G -47, G- 51), and yeast, *Candida* sp. (H-06, H-11). Lipase activities of *A. niger* ATCC 1015 were evaluated at temperature (25 – 60 °C), pH 5 - 9 and enzyme loading (10 -35 %, v/v) for optimization. The effect of inducers on lipase production was also carried out by using coconut oil, physic nut oil, groundnut oil and olive oil. *A. niger* ATCC 1015 gave the largest halo on the medium with 102.4 U/g activity. Zones of hydrolysis also increased with time and ranged from 3 mm to 10 mm at 30 °C for 96 h. The ability of cells to maintain sharp contrast between green medium and its clear zone without prior replication permits direct visualization and isolation of positive strains.

Optimum production of the enzyme (specific activity 216.7 Umg⁻¹) was attained at temperature 45°C, pH 7, and enzyme loading (25% v/v) with physic nut oil (2%) inducer. Hence *A. niger* ATCC 1015 strain can be commercially exploited as a potential lipase producing strain for industrial application.

Keywords: Bromocresol green medium; solid state fermentation; optimization; *Aspergillus niger* ATCC 1015

INTRODUCTION

Lipase (EC 3.1.1.3) occurs widely in nature and can be produced by many microorganisms, prokaryotes and higher eukaryotes. Lipases are present in higher plants seeds, such as castor bean and canola. In these plants, they are engaged in several lipid metabolism steps, including fat digestion, adsorption, and reconstitution. They are also found in several plants' energy reserve tissues (Sharma *et al.*, 2001; Castro *et al.*, 2004; Freire and Castilho, 2008; Jaeger and Eggert, 2002; Villeneuve, 2003; Cavalcanti *et al.*, 2007). Due to habitats' multiplicity, microorganisms usually produce various lipases types, with distinct specificity regarding to substrate utilization and also to optimum pH and temperature range. Lipases can be produced by bacteria, filamentous fungi, and yeasts, allowing these microorganisms to use lipids from animal or vegetable origin as carbon and energy sources for their growth. Though many microorganisms have been reported in literature as lipase producers, the genera *Candida*, *Rhizopus*, and *Pseudomonas* are considered the main industrial sources of lipases (Eastmond and Graham, 2001; FAO, 2010; Melo *et al.*, 2006). The yeast *Candida rugosa* is the most employed microorganism for lipase production (Freire and Castilho, 2008). Generally, microorganisms being the preferred source for the production of industrial enzymes have the following advantages, they have high yield of conversion of substrate into product, shortest generation time, simplicity in genetic manipulation and great versatility to environmental conditions.

The interest in lipase has grown over the last few years due to their diverse industrial applications such as additives in detergents, the elaboration of dietetic foods for use in the food industry, obtaining bioactive molecules in the pharmaceutical industry and pure optical compounds in chemical synthesis processes (Kazlauskas, 1994; Mohanasrinivasan *et al.*, 2009). Enzymes in organic media without a free aqueous phase are known to display useful unusual properties which have firmly established non aqueous enzyme systems for synthesis and biotransformations (Klibanov, 1997). Application of lipases in oleochemical processing saves energy and minimizes thermal degradation during

hydrolysis, glycerolysis, acidolysis and alcoholysis (Bornscheuer, 2000). Moreover, lipases have been successfully used as catalyst for synthesis of esters (Vulfson, 1994).

The use of solid media permits a fast screening of large populations of fungi, thus allowing the detection of specific enzymes (Bruhiman *et al.*, 1994; Thompson and Eribo, 1984; Zare-Maivan and Shearer, 1988) and helping in the chemotaxonomical differentiation of many microorganisms (Hankin and Anagnostakis, 1975). According to Cheetham (1987) the search for a new biocatalyst may require the screening of ten thousand to one hundred thousand microorganisms and an average of five to ten man years. Therefore, the emphasis on screening techniques rather than indirect or random methods should be on rational and direct screening procedures. Cheap, simple, rapid and very selective methods are of very great value. The use of lipases in industry is still limited by the cost of commercial enzymes, especially when large quantities of enzyme are required and when the final product is of low added value. There is therefore a considerable interest in reducing the cost of producing these biocatalysts.

The use of solid-state fermentation (SSF) as a production system is one way of reducing enzyme production costs, especially because agroindustrial waste can be used as a culture medium. Benjamin and Pandey (1997) cultivated *Candida rugosa* on coconut oil cake for lipase production using SSF and SmF systems. Enzyme yields were higher in the former. Several carbon sources- individually and in combinations were tested for their efficiency to produce lipases. Raw cake supported the growth and lipase synthesis by the yeast culture. However, supplementation with additional C- and N-sources increased enzyme titers. Bhusan *et al.* (1994) reported lipase production in SSF system from an alkalophilic yeast strain belonging to *Candida* sp. Rice bran and wheat bran, along with different concentrations of rice bran oil were used as the substrate. Rice bran supplemented with oil gave higher lipase yields.

We have adopted an innovative isolation technique which involves the use of artificial chromogenic substrates and selective media such as Bromocresol green agar for direct isolation of lipase producers from the groundnut cake waste substrates. Moreover, modified conventional SSF medium has been used for the

production and optimization of extracellular lipase of this screened *A. niger* ATCC 1015.

MATERIAL AND METHODS

Microorganisms

The fungal strains were isolated from groundnut cake wastes. Whole cakes were kept in the open air environment and fungi growths were noticed after a week. Pure culture of fungal isolates was obtained by sub culturing onto the Sabourand Dextrose Agar (SDA) medium. It was maintained on SDA slants at 4 °C.

Substrates

Rice bran, Soybean, cassava starch flour, olive oil and physic nut seeds were purchased from Itoku local market in *Abeokuta South LGA, Ogun State*. Physic nut and coconut oil was mechanically extracted using hydraulic pressed machine while groundnut oil was a gift from a friend in *Bida, Niger State, Nigeria*.

Media

Sabourand Dextrose Agar (SDA) by *Oxoid Ltd. England*.

Reagents

Bromocresol green dye (3',3',5',5'' Tetrabromo-m-cresolfonephthalein), Tween 80 (*Sigma Chemicals Ltd, USA*), acetic acid, (*BDH Chemicals Ltd., England*). Sodium hydroxide, sodium acetate, phenolphthalein (*Fluka, Switzerland*). All chemicals were of analytical grade (ANALAR).

Screening of lipase producing fungi

The bromocresol green agar medium prepared according to the method of **Akpan (2004)**. Pure culture of fungi isolated from one hundred and six (106) samples were inoculated on Bromocresol green agar medium containing Tween 80 and incubated at 30 °C for 72hrs. Lipase positive of fungal isolates produced colour change from green to yellow around the colonies while those that give no colour change were taken to be non lipolytic microorganisms (**Akpan 2004**). The diameter of the yellow zones around the colonies was estimated by metre rule taking the average of two perpendicular measurements.

Molecular characterization of fungi isolates

The extraction of total genomic DNA and Polymerase Chain Reaction (PCR) using standard methods were carried out at *Biotechnology Centre, Federal University of Agriculture, Abeokuta, Nigeria* while the sequencing was done at *Macrogen Inc. USA*.

Determination of enzyme activity

The isolates were cultured on solid state fermentation (SSF) containing rice bran, physic nut, cassava and soybean flour (5:5:1:3, w/w) according to the method of **Osho et al., (2014a)**. The crude enzyme was recovered from the moldy bran as described by **Mohanarivivasan et al., (2009)**. The lipase activity was determined according to the modified method of **Lin et al., (2008)**. Vegetable oil (8.9 ml) was treated with 1.0 ml of crude (prepared in 0.05M phosphate buffer, pH 7.0) and 0.1 ml of Tween 80 was used as emulsifying agent. The reaction mixture was mixed with vortex mixer for 1 min. It was then incubated in orbital incubator at 45 °C for 30 min at 180 rpm. The reaction was stopped by adding 25 ml warm ethanol to 1ml of the hydrolysate. It was further agitated manually for 1 min and titrated with 0.1 N NaOH until pH end-point 11.0 using phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme, which produced 1 μmol of fatty acids equivalent per minute under the assay conditions. The activity was established according to the following equation:

$$\frac{N.(V_s - V_b) . MM}{10.m}$$

Where

N = NaOH normality,

V_s = volume of base used in sample titration (ml)

V_b = volume of base used in blank titration (ml)

MM = Molecular Mass of the predominant fatty acid (g)

m = sample mass (g) **de Souza et al., (2010)**

Protein Determination

The concentration of protein in the enzyme was estimated by the method of **Groves et al., (1968)**. Protein content was determined by measuring the UV

absorbance of the sample at 260 nm and 280 nm. The method is based on the fact that nearly all proteins have an absorption maximum at about 280 nm (due mainly to tryptosine and tryptophan side chains), and a minimum near 260 nm whereas the reverse is true with nucleic acids. The protein concentration in sample is calculated using the following equation:

$$\text{Protein concentration (mgml}^{-1}\text{)} = (1.55A_{280} - 0.76A_{260}) \times \text{dilution factor}$$

Where A₂₈₀ absorbance at 280 nm

A₂₆₀ absorbance at 260 nm

$$\text{Specific Activity (Umg}^{-1}\text{ protein)} = \frac{\text{activity of lipase}}{\text{amount of protein loaded}}$$

Hama et al., (2007)

Characterization of Crude Lipase

Effect of temperature on lipase activity

The effect of temperature on lipase activity was determined at temperature range from 25-60 °C. The extract was buffered with 0.05 M phosphate buffer (pH 7) and vegetable oil was used as the substrate. The reaction mixture containing lipase (conc. 20%, v/v) was incubated in orbital incubator for 30 min at 180 rpm at each temperature. Enzyme activity was determined as described above.

Effect of pH on lipase activity

The lipase activity was evaluated at pH range from 5- 9 using different buffers (acetate buffer pH 5, phosphate buffer pH 6-9). The enzymatic extract was buffered with 0.05 M phosphate buffer at appropriate pH value and the reaction mixture containing lipase (conc. 20 %, v/v) and the substrate was incubated in orbital incubator for 30 min at 180 rpm at 45 °C. Lipase activity was determined as described above.

Effect of enzyme loading on lipase activity

The effect of enzyme loading on lipase activity was determined by varying the lipase concentration (10-35 %, v/v). The enzymatic extract was buffered with 0.05 M phosphate buffer (pH 6) and the reaction mixture containing lipase (at varying concentrations) and the substrate was incubated in orbital incubator for 30 min at 180 rpm at 45 °C. The activity was determined as described above.

Effect of different oils as inducer on lipase production

The effect of inducers on lipase production by *A. niger* ATCC 1015 was also carried out by substituting the Tween 80 with coconut oil, physic nut oil, groundnut oil and olive oil. These were added separately to the solid substrates and sterilized at 121°C for 15 min. The sterilized medium was allowed to cool and thereafter inoculated with 1.0 ml of 7 day old spores suspension of *A. niger* ATCC 1015 and incubated at 30°C for 96 h. The crude extract of fermented medium was assayed and the lipase activity and protein concentration were determined as described above.

RESULTS AND DISCUSSION

Screening of lipase positive fungi

Out of the one hundred and six (106) fungi isolated and screened on the Bromocresol green medium for lipase production, seven strains of mold and two of yeast were detected to be lipase positive. They were identified as *Aspergillus niger* [G-47, G- 51 (Figure 1), B- 17, B- 33]; *A. oryzae* [B- 05 (Figure 2)]; *A. niger* ATCC 1015 (Figure 3); *A. niger* CBS 513.88 with non lipolytic mold on Bromocresol green medium (Figure 4) and *Candida* sp. [H- 06 (Figure 5), H- 11]. The isolated lipolytic *A.niger* ATCC 1015 produced an extracellular lipase and its ability to hydrolyze lipid in the medium resulted in the colour change of the surrounding medium from green to yellow thus becoming acidic due to the release of fatty acids and glycerol. This was detected by adding Bromocresol green medium as the diameter of clear zones elaborated by the strains increased with incubation time. **Ogundero (1980)** established the fact that the lipolytic activities of fungi increased with increase in incubation period. Differences in the ability of the lipolytic fungi to hydrolyze oil could be due to the amount of enzyme excreted into the medium and the different growth rates of the strains.



Figure 1 Lipolytic strains of *Aspergillus oryzae* G-51 on the medium



Figure 2 Lipolytic strain of *Aspergillus niger* B-05 on the medium



Figure 3 Lipolytic strain of *Aspergillus niger* ATCC 1015 on the medium

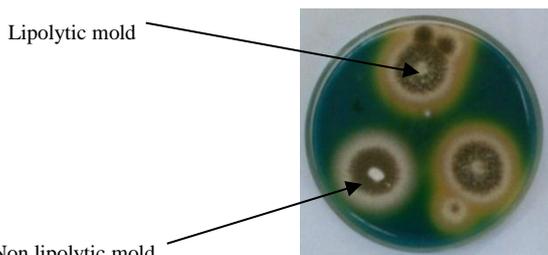


Figure 4 Lipolytic strain of *Aspergillus niger* CBS 513.88 and non-lipolytic mold on the medium

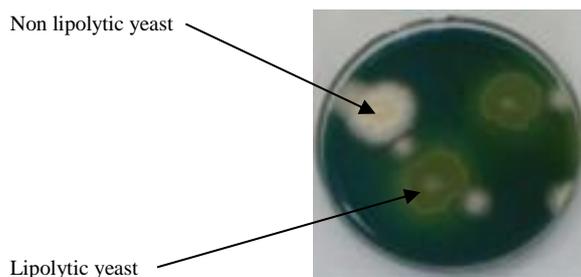


Figure 5 Lipolytic strain of *Candida* sp. H-06 and non lipolytic mold on the medium

Lipase production with zone of inhibition by various strains of microorganisms isolated on the substrate

Table 1 shows lipase production by strains of *Aspergillus niger* and *A. oryzae*. *A.niger* CBS 513.88 gave lipase activity of 52.7 Ug⁻¹ with 5mm diameter of yellow zone around the colonies at 72 h incubation time among the isolated species while *A. oryzae* G- 51 had 34.8 Ug⁻¹ with 4 mm width of clear zone at 96 h incubation time. *A. niger* ATCC 1015 (Figure 3) gave the highest lipase activity of 102.4 Ug⁻¹ with 10 mm width of clear zone at 96 h incubation time which is 2.94-fold increase compared with the *A. oryzae* G-51. The lipase producing *Candida* sp. strains H- 06 had lipase activity of 15.3 Ug⁻¹ with 3 mm width of clear zone at 96 h incubation time as compared with its counterpart strain H- 11 with 14.4 Ug⁻¹ and 4 mm diameter of yellow zone around the colonies at the same incubation time (Table 2). The lipolytic activity demonstrated by *A. niger* ATCC 1015 was 6.7 times higher than the best yeast strain. Figure 5 showed *Candida* sp. H- 06 with non lipolytic white colony. In all isolates, the diameter of clear zones elaborated by the strains increased with incubation time. Loss of activity on the fourth day of incubation may be attributed to the deactivation of the enzymes as a result of slight acidic medium (not reported). This result support the findings of Osho et al., (2014b) when extracellular lipase was synthesized from a rice-bran phytic nut cake waste as solid substrates. Lipases have been produced from different microorganisms using SSF with different solid wastes, such as lipase from *Penicillium restrictum* in babassu cake (Azeredo et al., 2007); lipase from *Candida rugosa* in rice flour (Rao et al., 1993). These lipases were produced by SSF on a bench scale, mostly using tray bioreactors, and yielded high productivity rates.

Table 1 Lipase production by *Aspergillus niger* and *A.oryzae* strains

Strains	Width of clear zones (mm) ^o				Lipase activity (Ug ⁻¹)*
	24 h	48 h	72 h	96 h	
<i>A.niger</i> CBS 513.88	2	4	5	5	52.7 ± 0.83
<i>A.niger</i> B- 17	1	3	4	5	24.4 ± 0.64
<i>A.niger</i> B- 33	1	2	2	4	15.3 ± 0.12
<i>A.niger</i> ATCC 1015	3	6	8	10	102.4 ± 0.91
<i>A.niger</i> B-05	2	4	6	7	43.6 ± 0.16
<i>A.oryzae</i> G- 47	2	3	3	4	22.7 ± 0.67
<i>A.oryzae</i> G- 51	2	3	4	4	34.8 ± 0.22

*Mean of triplicate ± standard deviation

^oZone of hydrolysis is the average of two perpendicular measurements

Table 2 Lipase production by *Candida* sp. strains

Strains	Width of clear zones (mm) ^o				Lipase activity (Ug ⁻¹)*
	24 h	48 h	72 h	96 h	
<i>Candida</i> sp. H-06	1	2	2	3	15.3 ± 0.33
<i>Candida</i> sp. H-11	1	2	3	4	14.4 ± 0.14

*Mean of triplicate ± standard deviation

^oZone of hydrolysis is the average of two perpendicular measurements

Characterization of crude lipase of *A. niger* ATCC1015

Effect of assay temperature on lipase activity

The effect of assay temperature on lipase activity of the crude enzyme is presented in Figure 6(a). Temperature had great influence on the activity of the enzyme. As the temperature increases the activity increases with optimum activity of 156.7 Ug⁻¹at 45 °C and decreased drastically at temperatures above 60 °C. The basis for stability of lipase at relatively high temperature might be linked to the fact that the lipases are highly hydrophobic (Pandey and Benjamin, 2001). The hydrophobic amino acids are concentrated near the catalytic center with the non-polar surface around the active site greatly expanding thus the amino-acids found on the side of the 'lid' that overarching the catalytic site (Ser-His-Glu) become completely exposed (Gordillo et al., 1995). However, Adebisi

et al., (2003) attributed the thermostability properties of the lipase to the presence of allosteric inhibitors which protect the enzyme against heat inactivation. Similarly, lipases from *A. niger* strains have been reported to be active between 40 and 55 °C (Kamini et al, 1998; Namboodiri et al., 2000; Falony et al., 2006).

Effect of assay pH on lipase activity

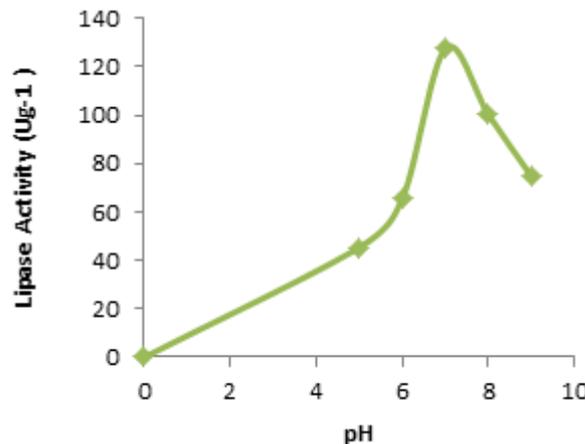
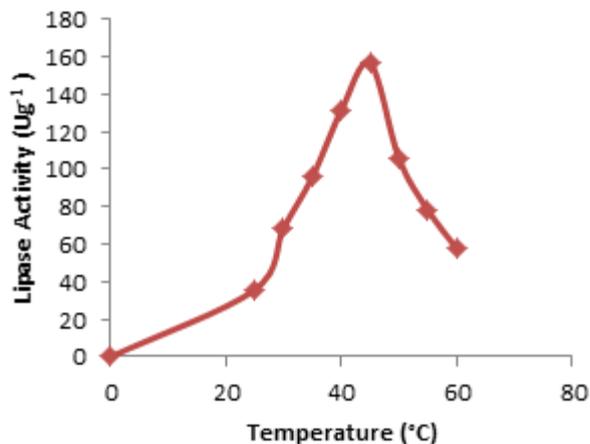
The result of pH studies as shown in Figure 6(b) indicated that the crude lipase extract was most active at pH 7 with optimal activity of 127.7Ug⁻¹. However, it could also exhibit activeness in slightly alkaline medium. **Crueger and Crueger (1993)** and **Falony et al., (2006)** obtained similar result for a lipase expressed by another *A. niger* strain. The gradual pH increase was probably caused by the release of proteases resulting in deamination of amino acids and liberation of ammonia (**Gombart et al., 1999**). However, the stability studies of *Candida rugosa* lipase conducted by **Pandey and Benjamin (2001)**, focused mainly on the conformational changes caused by the pH changes. That change might reduce or impose strain on the 'lid' overarching the active center, thereby open or shut down the catalytic center for substrate binding.

Effect of enzyme loading on lipase activity

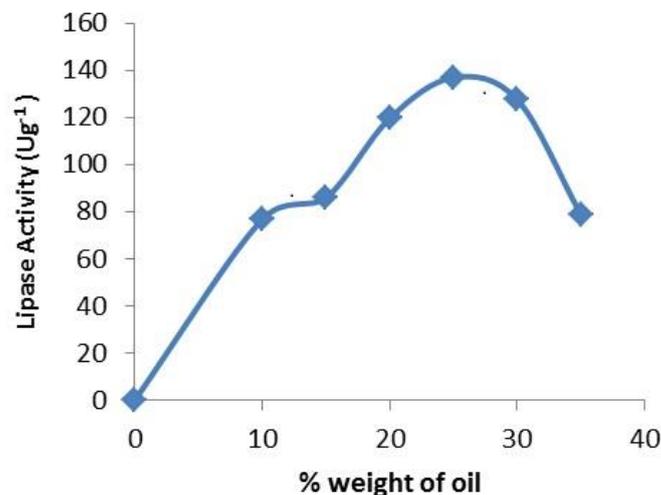
The effect of enzyme loading on the lipase activity the enzyme is presented in Figure 6(c). The enzyme loading between the ranges 20 – 30 % weight of oil substrate slightly increases the production of lipase. Thus enzyme loading had an insignificant effect on lipase production (P ≤ 0.05). The optimal loading of 25% (w/v) gave the maximum activity of 136.9 Ug⁻¹ which retained about 75 % of its initial activity at 30 °C. Similar results have been reported for other fungal lipases by **Mozaffar and Weete (1993)** and **Phillips and Pretorius (1991)**.

The effect of different vegetable oils on lipase production

The effect of inducers on lipase production is shown in Figure 7. The substitution with physic nut oil significantly increases lipase production with specific activity at 216.7Umg⁻¹ while groundnut, coconut and olive oil is 157.6 Umg⁻¹, 86.8 Umg⁻¹ and 64.7 Umg⁻¹ respectively. Analysis of variance showed that the influence of these vegetable oils used as inducers is significant at 5 % level. The findings on the effect of inducers on lipase production which revealed that physic nut oil (2 %) has the highest enzyme specific activity can be established by the result of **Rao et al. (1993)** that verified that oil content was a significant variable affecting the yield. **Ban et al. (2001)** also in their work concluded that oil acted as carbon source as well as inducers for lipase production. Extracellular lipase production by different microorganisms on lipids has been extensively reported (**Nutan et al., 2002**).



(a) (b)



(c)

Figure 6 Effect of assay temperature (a); pH (b) and enzyme activity (c) on lipase activity of *A. niger* ATCC 1015

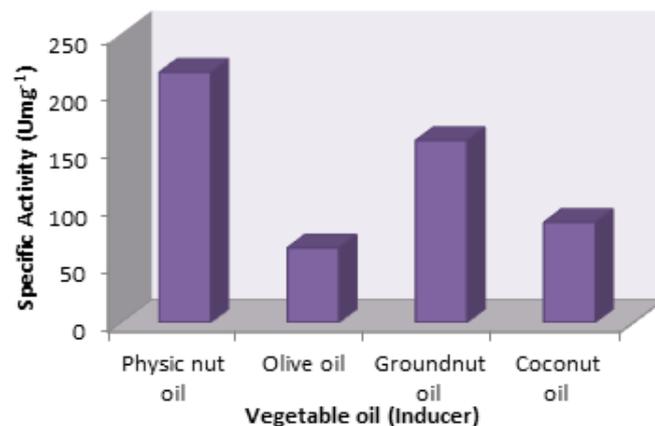


Figure 7 Effect of different vegetable oils as on lipase production

CONCLUSION

This extracellular lipase from strain *A.niger* ATCC 1015 is of industrial and commercial interest and can be synthesized by simple screening in a chromogenic substrate without necessarily interfering cell intact with maximum

productivity. Increase in temperature increases the activity with optimum 156.7 U_g⁻¹ at 45 °C. The lipase extract was most active at pH 7 with optimal activity of 127.7 U_g⁻¹ and enzyme loading between 20- 30 % weights of oil substrate slightly increases the production with maximum activity of 136.9 U_g⁻¹ which is about 75 % of its initial activity. The inducer substitution with physic nut oil significantly increases lipase production with specific activity of 216.7 U_{mg}⁻¹.

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