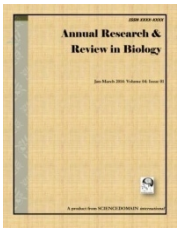

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Screening and Optimization of Process Parameters for the Production of Lipase in Submerged Fermentation by *Aspergillus carbonarius* (Bainer) IMI 366159

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerol at the oil-water interface to release glycerol and free fatty acids. In this study culture conditions and nutrient source modification studies were carried out on lipase production by *Aspergillius carbonarius.*
Study Design: The work was based on a completely randomized design for the process parameters. All the experiments were conducted at least twice and the analyses were carried out in triplicates and average values were calculated for mean comparison.
Methodology: The fungus was initially screened for the production of lipase using Rhodamine B agar media. Lipase production studies were conducted in a basal medium. Thereafter, the influence of carbon, nitrogen, pH, temperature and metal ions were examined on both the growth of fungus and its lipase production by monitoring the rate of olive oil hydrolysis by titration.
Place and Duration of Study: The present study was conducted at the Food and Industrial Microbiology laboratory, Department of Microbiology, University of Port Harcourt, Nigeria between February 2012- October 2013.
Results: Lipase production was detected as an orange halo around the fungal colonies under UV light at 350nm.The evaluation of suitable carbon and nitrogen sources for lipase

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production revealed that glucose (2%) and peptone (2%) yielded high lipase production. Olive oil (1.5%) was the best triglyceride (inducer) while the most suitable trace element for maximum lipase production was zinc sulphate (0.05%) followed by magnesium sulphate and manganese sulphate. Under optimal culture conditions, maximum lipase production (1.3 U/ml) was obtained in 96 h at 30ºC and pH 6.0 using 2% glucose as carbon source and 2% peptone as nitrogen. The correlation analysis carried out on all the experiment proved the interdependent of the physicochemical parameters and lipase production in *A. carbonarius.*

Conclusion: The result obtained in this study indicated that *A. carbonarius* is a good producer of extracellular lipase; thus, making the fungus attractive for potential biotechnological applications.

Keywords: Aspergillus carbonarius; lipase; screening; fermentation; optimization.

1. INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a class of hydrolases that are primarily responsible for the hydrolysis of triacylglycerol at the oil–water interface to release glycerol and free fatty acids [1-3]. They are ubiquitous and indispensible in nature and are produced by animals, plants and microorganisms [2,4,5]. Microbial enzymes are often more useful than the enzymes derived from plants and animals due to the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply because of the absence of seasonal variations and rapid growth as well as being more stable and more convenient [4]. Microbial lipases are very prominent biocatalysts due to their potential to catalyze a wide variety of reactions in aqueous and non-aqueous media, including hydrolysis, inter-esterification, alchoholysis, acidolysis, esterification and aminolysis [2,3,6]. Microbial lipases are an important group of technologically relevant enzymes and they find immense applications in dairy, food, laundry detergents and pharmaceutical industries [4]. Novel biotechnological applications have successfully established the use of lipases in the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceutical, agro-chemicals and flavour compounds [3,7]. These wide range of utilization of lipases have increased interest in the discovery of new sources of the enzyme with suitable property to suit specific applications.

Lipases occur widely in bacteria, yeasts fungi and actinomycetes [1,3,8-22] and differ enormously with regard to both their origin and their properties. Due to huge variation in usage, the availability of lipases with specific and unique characteristics is still a limiting factor. Hence, to search for new lipases with different characteristics and improve lipase production continues to be important research topics [13]. In view of this, access to a wider range of lipase types with properties suitable for different applications would be beneficial [23]. *Aspergillus carbonarius* (Bainer) IMI 366159 originally isolated from rotten cassava is known to produce both raw starch digesting amylase and protease with novel characteristics [24,25]. In fermentation process, lipase production is mainly affected by carbon and nitrogen sources, agitation, and dissolved oxygen concentration among others. Lipases are mostly inducible enzymes and inducers such as oils are important for lipase secretion [26]. The present study was undertaken to optimize the conditions for lipase production from *Aspergillius carbonarius* (Bainer), IMI 366159.

2. MATERIALS AND METHODS

2.1 Microorganism and Growth Conditions

Aspergillus carbonarius (Bainer) IMI 366159, originally isolated from rotten cassava tuber [27] was used for this study. The culture was maintained on potato dextrose agar (PDA) slants at 4ºC and subcultured every 6 months.

2.2 Materials

Analytical reagent grade chemicals were purchased from commercial sources at the highest purity, unless otherwise mentioned.

2.3 Rhodamine B agar Screening

The primary screening for the detection of lipolytic activity was carried out on rhodamine B agar (RBA) as described by [1] with some modifications. The growth medium, PDA was adjusted to pH 6.0, autoclaved and cooled to 60ºC. Then, filter-sterilized rhodamine B stock solution (1.0 mg/ml) in distilled water was added to a substrate lipoidal emulsion to yield a final concentration of 0.001% (w/v). The substrate lipoidal emulsion consisted of 1.5% (w/v) olive oil with 0.25% (v/v) Tween 80 in distilled water that was sterilized by autoclaving. The resulting mixture of lipoidal emulsion with growth medium (1:10) was vigorously stirred to emulsify for 15 min. The medium was allowed to stand for 10 min at 60ºC to reduce foaming before pouring 20 ml of medium into plastic petri plates. Fresh RBA plates were spot inoculated with 72 h-old spores of *Aspergillus carbonarius* in sterile saline and incubated at 37ºC for 6 days. The plates with visible growth were UV irradiated (350 nm). Lipase production was identified as orange fluorescence under UV light.

2.4 Microorganisms and Culture Conditions

Suspensions of mature spores of *Aspergillus carbonarius* were obtained by gently washing the surface of agar slants with 5ml sterile saline. The composition of basal medium used was (g/l) NH_4NO_3 2, sucrose 20, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 2; $CuSO_4.7H_2O$ 0.06. The pH was adjusted to 6.0 with 1M NaOH or 1 M HCl. Thereafter, 1% (v/v) olive oil was added. Media were sterilized for 15 min at 121ºC at 15 psi. The sterilized medium was inoculated with 5 ml inoculum of the fungal spores and incubated in Erlemeyer flask containing 50 ml of basal medium on a rotary shaker at 120 rpm for 72 h at 30ºC. After 72 h, samples were filtered in a double layered muslin cloth followed by Whatman No. 1 filter paper. The clear supernatant was used for lipase activity assay.

2.5 Lipase Assay

Lipase activity in the clear supernatant was determined titrimetrically by the olive oil substrate emulsion method [28]. The reaction mixture consisted of 1.0 ml of the substrate emulsion (70.0 ml emulsifying reagent with 30.0 ml olive oil) homogenized for 5 min. The emulsification reagent (NaCl 17.9 g, KH_2PO_4 0.41 g, glycerol 540.0 ml, gum arabic 10.0 g and distilled water to a total volume of 1.0 L), 0.8 ml of 0.2 M potassium phosphate buffer (pH 7.0) and 0.2 ml of the enzyme were incubated at 37ºC for 30 min. The reaction was terminated by adding 2.0 ml of acetone-ethanol mixture (1:1 v/v). The amount of fatty acid liberated was determined by titration with 0.01 N NaOH. One unit of lipase activity was

defined as the amount of enzyme required to release 1 µmol of fatty acid per ml per min under the above assay conditions. All fermentations and assays were carried out in triplicate and the mean values were presented.

2.6 Dry Cell Weight Determination

In order to determine the fungal biomass, the mycelium was filtered through filter paper (Whatman No. 1). The filtered mycelium was washed with distilled water and dried at 105ºC to constant mass. It was then placed in the desiccator and the weight determined.

2.7 Optimization of Medium Parameters

The medium parameters optimized were carbon source, nitrogen source, metal ions, effect of triglycerides, temperature and incubation time. The selection of carbon sources, nitrogen sources, metal ions and triglycerides were based on earlier reports of increase in lipase activity in other microorganisms especially fungi. The strategy adopted in the optimization of medium components was to optimize one particular parameter at a time and then include it at its optimum value in the next optimization step. The general procedure for cultivation was followed as earlier described. Each experiment was carried out in triplicate and average value presented.

2.8 Effect of Carbon Source Additives on Lipase Production

In order to determine the effect of carbon additives on lipase production, sucrose in the basal medium was substituted individually with 1% (w/v) of the following sugars in the presence of olive oil: glucose, maltose, fructose, lactose and manitol and starch. Different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4%) of the best carbon source were then evaluated to determine the most favourable concentration for optimum lipase production.

2.9 Effect of Nitrogen Sources on Lipase Production

To evaluate the effects of organic and inorganic nitrogen sources, NH_4NO_3 was substituted with peptone, yeast extract, urea, sodium nitrate, ammonium chloride, ammonium sulphate and tryptone each at 0.5% (w/v). Thereafter, the influence of different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0%) of the best nitrogen source was evaluated to determine the level of lipase production.

2.10 Effect of Triglycerides

Triglycerides and fatty acids have been reported to induce lipase secretion in various microorganisms [4]. The effects of different triglycerides were evaluated by individually incorporating the following triglycerides in place of olive oil at a concentration of 1% (w/v): palm oil, groundnut oil, sunflower oil and soybean oil. After which different concentrations (0.5-4.0%) of the best triglycerides were evaluated to assess the optimum concentration for lipase production.

2.12 Effect of Metal Ions on Lipase Production

The effect of various metal ions was assessed on lipase production by individually including them in the basal medium. The following metal ions were tested at concentration of 0.05%

(w/v): calcium chloride, zinc sulphate, manganous sulphate and ferrous sulphate. Medium without trace elements was used as a control.

2.13 Effect of Initial pH and Temperature

The effect of initial pH and incubation temperature on the culture was evaluated at different pH (3.0-9.0) using different buffer solutions and varied temperature range (20-50ºC), respectively.

2.14 Time Course of Lipase Production

The amount of lipase produced was determined daily for a period of six days in the formulated medium with the best parameters for lipase secretion. Samples were analysed at 24 h intervals to determine the pH, dry biomass and lipase activity in the supernatant.

2.15 Statistical Analysis

Data obtained were analysed using analysis of variance with SPSS version 15.0. The level of tested significance was at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

Aspergillus carbonarius Bainer IMI 366159 was screened for lipase using Rhodamine B agar medium. A brilliant pink-red/orange fluorescent zone on Rhodamine B agar plates was observed under UV irradiation (350 nm), indication that the fungus was able to hydrolyze olive oil. Lipase screening in basal medium in Erlenmeyer flask showed the presence of lipolytic activity in cell-free filtrate prepared from *A. carbonarius* culture broth. Most extracellular production of microbial enzymes is influenced by the conditions of cultivation [11,29].

3.1 Effect of Carbon Source Additives on Lipase Production

Lipase production by *Aspergillus carbonarius* (Bainer) IMI 366159 was significantly increased with the incorporation of sugars to the basal medium in some cases. Fig. 1 showed the effect of different sugars on lipase production. The data obtained indicated that the addition of glucose gave the highest lipase activity (1.25±0.03 U/ml) followed by sucrose (1.02±0.02 U/ml). Maltose and fructose as carbon source additives were found to be comparatively good in enhancing lipase production. Manitol, starch and lactose significantly repressed lipase secretion (0.31±0.04 U/ml, 0.17±0.02 U/ml and 0.14±0.01U/ml, respectively). The effect of different concentrations of glucose (best carbon additive) on lipase production is presented in Fig. 2. The result revealed that different concentrations exerted significantly varied effects on lipase production. The highest lipase production (1.2±0.02 U/ml) was observed at 2% glucose concentration. A further increase of glucose level beyond 2% decreased lipase production. Many researchers have reported the positive effect of sugars on lipase production [30-32]. The results of this study with regard to effect of carbon sources on lipase production indicated that glucose supplementation in the culture medium enhanced lipase production. This result is in contrast with the report of a study carried out by other researchers [33,34]. However our result is similar to the report of [35] who observed that starch, mannitol and lactose led to decrease in lipase production while fructose encouraged lipase production. Other workers have obtained maximum lipase

secretion with 2% glucose for some fungal strains which agreed with our findings [36]. However, the findings of the present study is consistent with the report of [19] which stated that the best lipase activity was obtained when olive oil and glucose were added to the medium at 2% concentration, respectively. Similarly, 2% glucose was reported to produce significant increase in lipase production by *Yarrowia lipolytica* NCIM 3589 [21].

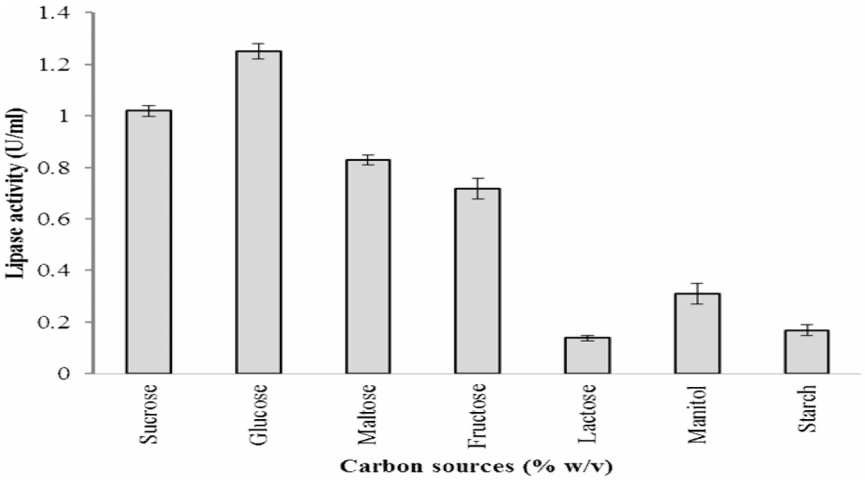

Fig. 1. Effect of carbon sources on lipase production

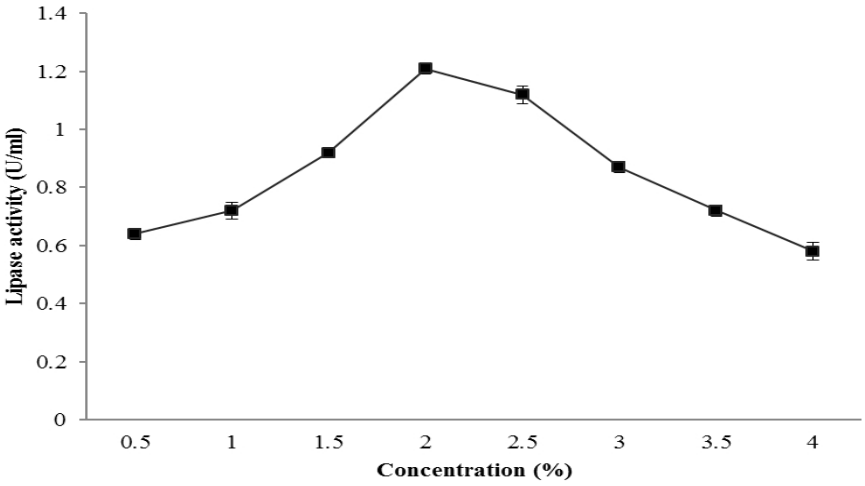

Fig. 2. Effect on different concentration of glucose on lipase production

3.2 Effect of Nitrogen Sources on Lipase Production

Fig. 3 depicted the effect of various nitrogen sources on lipase production. Lipase production was found to be highest with peptone (0.92±0.03 U/ml). Urea gave the lowest lipase (0.25±0.03 U/ml) yield followed by tryptone (0.25±0.01 U/ml) and yeast extract (0.43±0.01 U/ml). The effect of different inorganic nitrogen sources showed ammonium sulphate

(0.84±0.04 U/ml) as the best inorganic nitrogen source followed by ammonium nitrate (0.78±0.02 U/ml).

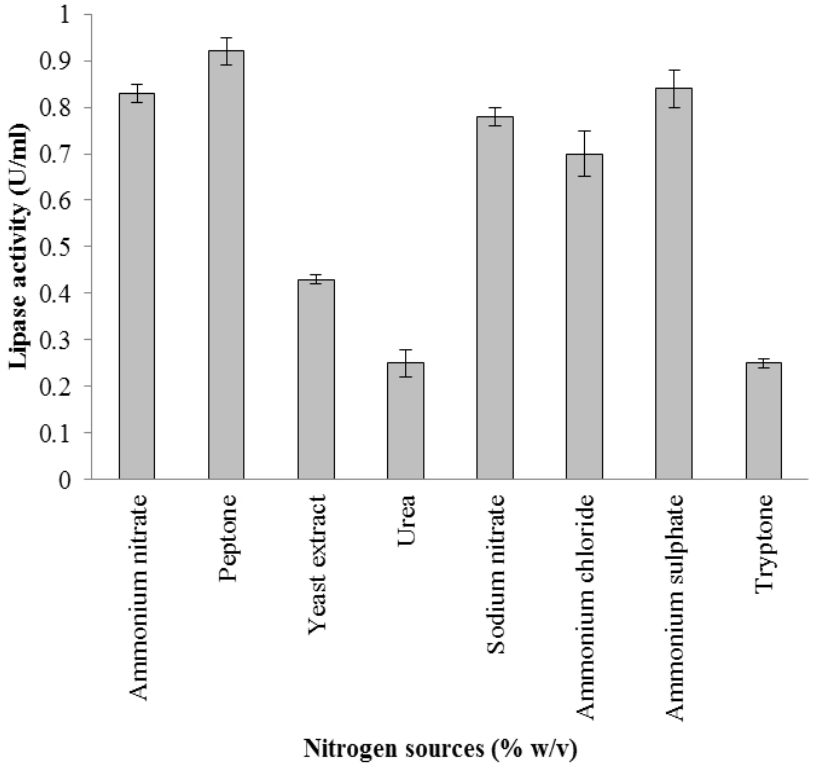

Fig. 3. Effect of nitrogen sources on lipase production

The data of Fig. 4 showed that the effect of different amounts (0.5 – 0.4%) of peptone on the production of lipase. Maximum lipase production (0.88±0.02 U/ml) was observed when 2% peptone was used in the fermentation medium. Further increment in the amount of peptone led to decline in lipase production. Other studies have shown the use of 2% corn steep liquor for the production of lipase by fungal strains [35]. This observation is in contrast with our findings where 2% peptone was the best in enhancing lipase production in *A. carbonarius*. Microorganisms have been reported to provide high yields of lipase when organic nitrogen sources are employed [37]. However, our result is similar to workers [38-40]. This finding agrees with the results of another study in which 2% peptone was the best nitrogen source for maximum lipase production by some fungal strains [35].

3.3 Effect of Triglycerides as Inducers

The important role of different triglycerides on lipase production by this fungus was elucidated by individually adding the selected triglycerides (1%, v/v) to the culture medium. Five different natural oils were tested Fig. 5. All the lipid substrates enhanced lipase production ranging from 0.63±0.03 U/ml – 0.81±0.01 U/ml. The highest lipase production (0.81±0.01 U/ml) was obtained with olive oil. The next best lipid sources observed were groundnut oil (0.74±0.02 U/ml) and soybean oil (0.72±0.02).Among the natural oils tested,

palm oil gave the lowest lipase activity (0.63± 0.03 U/ml).In the present study, the effect of different concentration of olive on lipase production showed a positive linear increase (0.64±0.03 – 0.98±0.01 U/ml) as the concentration of olive oil increased from 0.5-1.5% (v/v) Fig. 6. A further increase in concentration of olive oil above 1.5% led to a decline in lipase production from 0.98±0.01 U/ml to 0.52±0.02 U/ml. The maximum lipase production was obtained at 1.5% concentration of olive oil. Olive oil and Sunflower oil have been reported to increase the yield of lipase in other microorganisms [21]. The finding of our study on the addition of lipids in the fermentation medium is in agreement with the report of other workers [10,36-38].

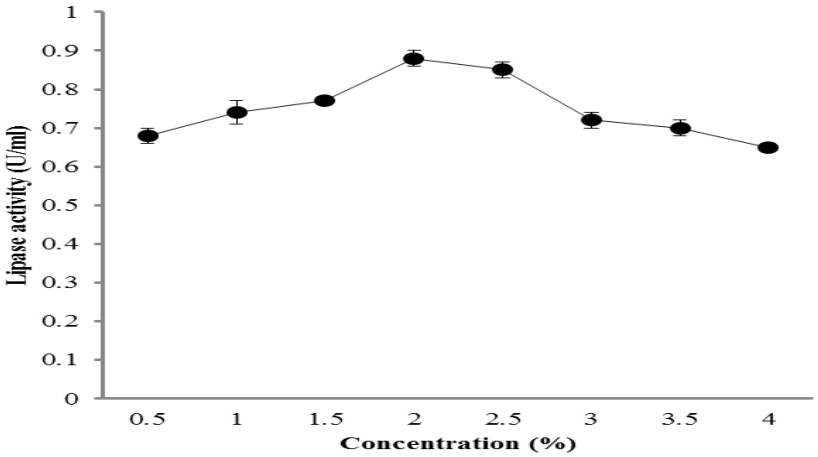

Fig. 4. Effect of different concentration of peptone on lipase production

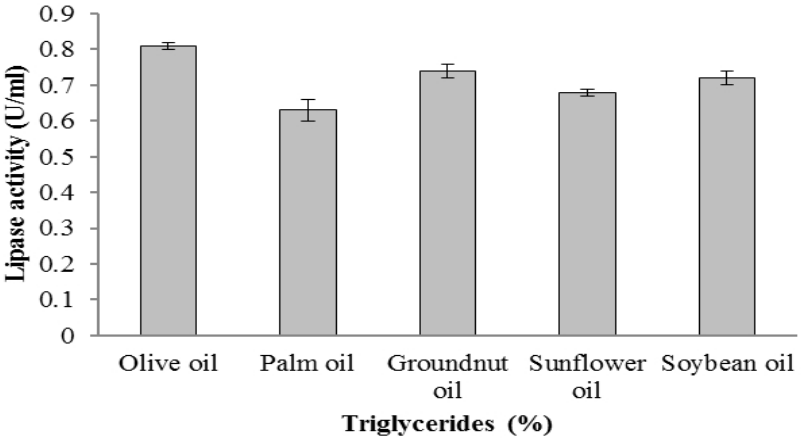

Fig. 5. Effect of triglycerides on lipase production

3.4 Effect of Metal Ions on Lipase Production

The effect of different metal ions on lipase production is shown in Fig. 7. The result revealed that all the trace elements enhanced lipase production. Zinc sulphate induced the highest lipase production (0.84±0.02 U/ml), followed by magnesium sulphate (0.78±0.02 U/ml). The lowest lipase production was obtained with calcium chloride (0.63±0.03 U/ml). Divalent ions of magnesium showed maximum lipase production in *Metarhizium anisopliae* whereas

divalent metal ions of ferric inhibited lipase production [38]. Conversely, manganese was reported as the best metal ion for maximum production of lipase by some fungal strains [34].

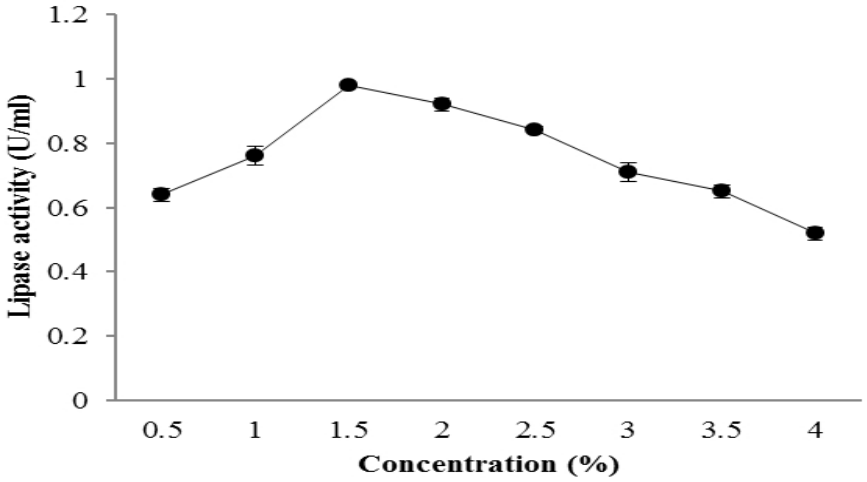

Fig. 6. Effect of different concentrations of olive oil on lipase production

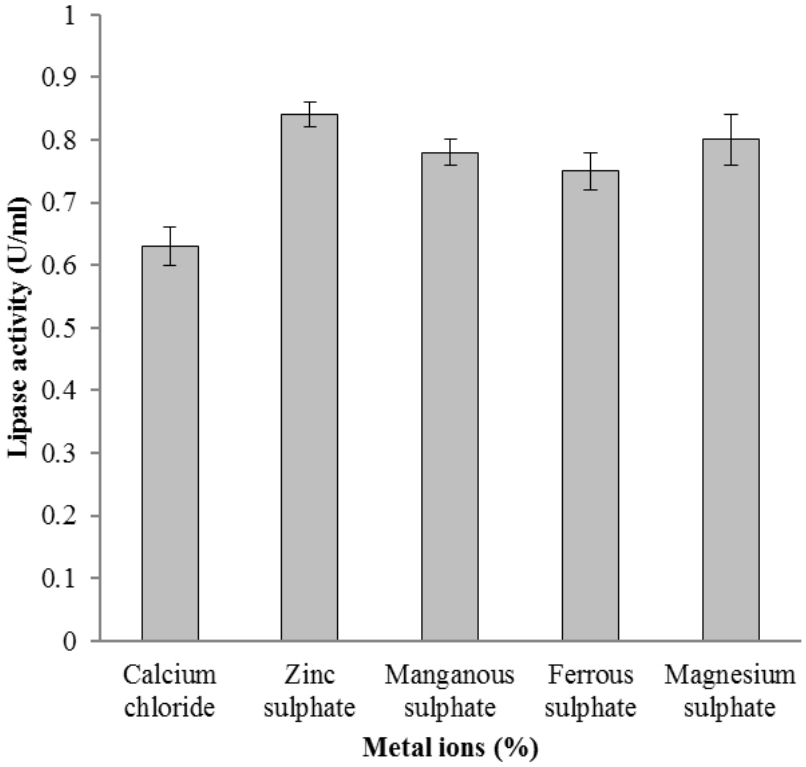

Fig. 7. Effect of metal ions on lipase production

3.5 Effect of Initial pH and Temperature

Fig. 8 showed the effect of initial pH on lipase production. The initial pH of the culture was found to be one of the most critical environmental parameters influencing enzyme production by *Aspergillus carbonarius*. Maximum lipase production resulted at an initial medium pH of 6.0. Lipase production significantly ($p \leq 0.05$) decreased at alkaline and acidic pH of 9.0 and 3.0, respectively. The initial pH of the growth medium is essential for lipase production [1]. Lipase activity and growth were significantly affected by the initial pH. Our result is consistent with the findings of [35] who reported that the optimum pH for lipase production by two fungal strains was at pH 6.5, respectively. Also, our result is similar to [36]. Most available reports for the production of lipase by fungi are conducted under acidic conditions [41,42]. Furthermore, [38] reported that maximum lipase secretion by *Metarhizium anisopliae* was at pH 5.7 whereas pH of 6.5 has been observed for *Aspergillus niger* [43]. In variance with our findings, [40] reported that maximum lipase production by *Aspergillus* species was obtained at pH 7. Similarly, an initial pH 6.0 was reported as the optimum pH for lipase production by strains of *Aspergilli* [44].

Submerged fermentation was carried out at 20-50ºC to evaluate the effect of incubation temperatures on lipase production. The effect of temperature on lipase production is presented in Fig. 9. Maximum lipase production was observed at temperature of 30ºC. Data obtained revealed that incubation at temperatures below 30ºC and above 30ºC significantly ($p \leq 0.05$) decreased enzyme production. Maximum temperature of 30ºC has been obtained as the best temperature for the production of extracellular lipase by two fungal strains [34]. In contrast to our result, maximum lipase production by some fungal isolates occurred at 26ºC [36]. Our result is consistent with the findings of [38] who reported maximum lipase production by entomopathogenic *Manisopliae* at a temperature of 32ºC. Our finding is similar to the reports of [45]. They found that *Penicillum notatum* and *Fusarium oxysporum* produced maximum lipase at 30ºC. In another vein, our report differed with the report of [43] who obtained maximum lipase production by *A. niger* at temperature of 37ºC. Maximum lipase production has been reported for *Yarrowia lipolytica* at 30ºC [21], which also agreed with our findings.

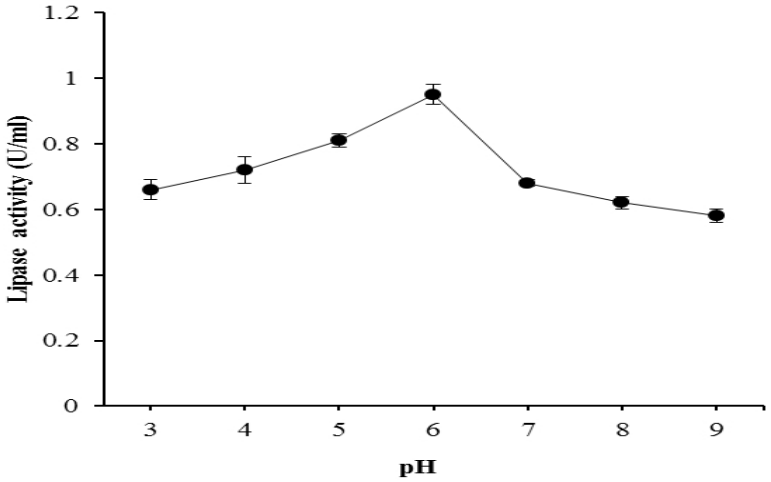

Fig. 8. Effect of initial ph on lipase production

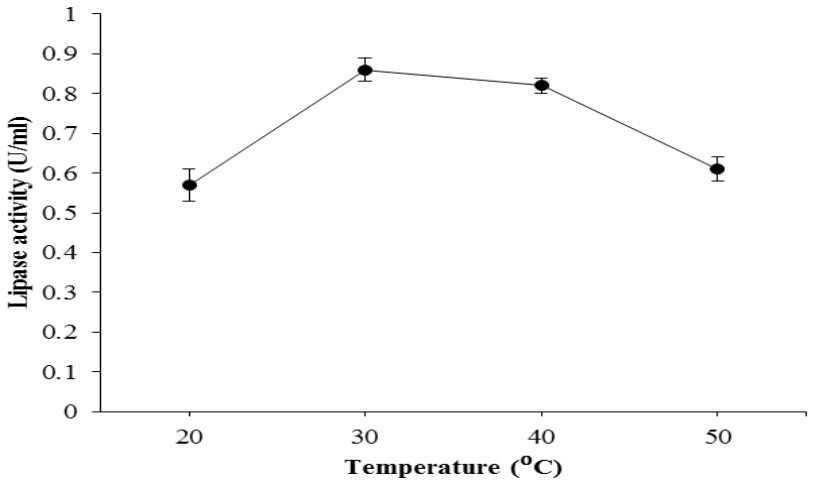

Fig. 9. Effect of temperature on lipase production

3.6 Time Course of Lipase Production

The time course of lipase synthesis in the modified medium by *A. carbonarius* was monitored by measurement of lipase activity, dry biomass and pH Fig. 10. The modified culture medium for lipase production by this fungus consisted of 1.5% olive oil (v/v), 2% (w/v) glucose, 2% (w/v) peptone, 0.05% (w/v) zinc sulphate, initial pH 6.0 and the culture incubated at 30ºC for 144h. Lipase production commenced at 24h and reached a maximum at 96h. However, a further increase in incubation time did not lead to corresponding increase in lipase activity Fig. 10. The result indicated that there was corresponding shift in pH from 6.0 to 5.7 during the first 24h of incubation decreasing to 4.5 at 144h. Maximum enzyme activity was obtained when the biomass was the highest (2.3mg/ml) at 96h and the final culture pH was 4.6.It has been reported that lipase production increase as incubation time increases but later falls with the increase in incubation period [46]. This trend in lipase production could be attributed to proteolytic degradation of the enzyme system [24]. Our results is in agreement with the studies carried out by [35] who observed that maximum lipase was secreted at 96 h (4 days) of incubation period by two fungal strains (TP. St. 02 and TP. St. 05). Moreso, similar results have been reported in several studies involving fungal isolates [47]. Maximum lipase production by *Penicillum verracosum* occurred at 96 h [48]. In addition, our result is consistent with other reports [39]. Our observation differed with the report of [49] which asserted maximum lipase production by some fungi at 72 h. Our result also differed with report on *P. notatum*, *Macrophomina phaseolina* and *Rhizopus stolonifer* where a longer day of incubation period (25 days) was obtained for maximum lipase production [45].

Moreover, in *A. niger*, maximum lipase occurred at the 4th day of incubation which is similar to our findings [43]. In another report, [50] asserted that maximum production of lipase by *A. niger* occurred on the 6th day of fermentation. Hence, this observation differs with our present finding. Another contrast reported was obtained by [51] who affirmed that maximum lipase production by *Aspergillus* sp and *Trichoderma* sp occurred at day 7 and 14, respectively. In contrast to our findings, maximum lipase production was attained on day 5 of incubation period for strains of *Aspergilli* [44]. Also, *Yarrowia lipolytica* secreted maximum lipase production on the 4th day of fermentation [21]. This agrees with the present findings.

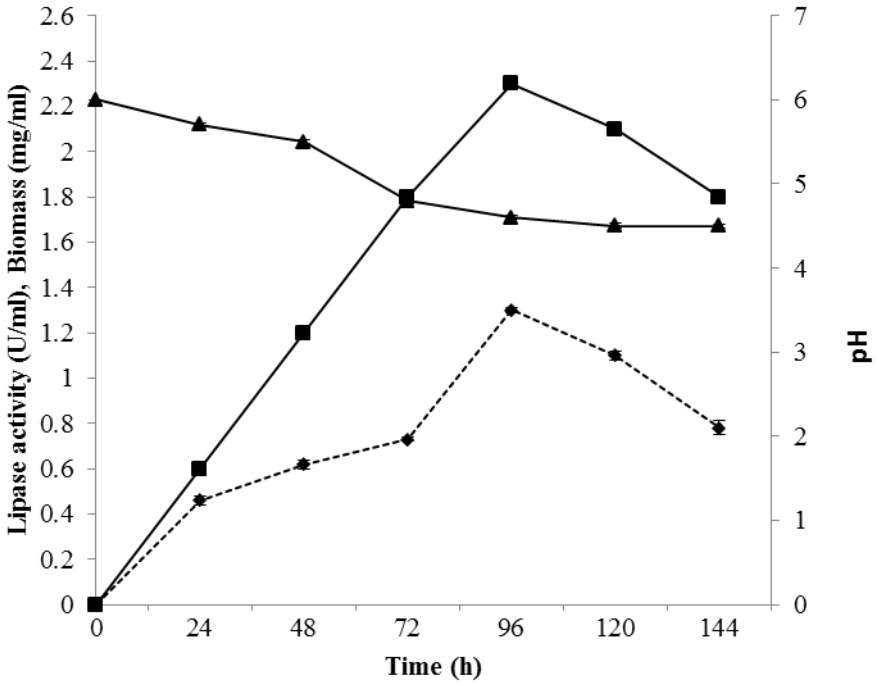

Fig. 10 Time course of lipase production by *A. carbonarius*. Formulated medium (best medium parameters) was seeded with 5% (v/v) inoculum incubated at 30^{0}C for 144 h at 120 rpm. samples were withdrawn at 24 h intervals to monitor biomass, pH and lipase act

4. CONCLUSION

In this work, the ability and optimal conditions for the production of extracellular lipase by *Aspergillus carbonarius* were determined. The various nutritional and environmental parameters such as carbon and nitrogen sources, induction by lipids, incubation time, initial pH and temperature were optimized. This present study identifies *A. carbonarius* as a producer of extracellular lipase and under optimal condition of the process parameters, a significant increase in lipase yield was achieved. The correlation analysis conducted on all the results of the experiment revealed the interdependence of the physicochemical parameters and lipase production in the fungus. This provides essential basic information to achieve the large scale production of lipase by *A. carbonarius*. Due to the non-complex and inexpensive nature of the optimized medium in this study, the fungus and the new extracellular lipase could have great biotechnological potential. However, further study which is ongoing in our laboratory is essential to purify and characterize the lipase produced by *A. carbonarius* in order to understand its biochemical properties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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