Screening and Optimization of Submerged Fermentation of Lipolytic *Aspergillus oryzae*

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Lipases are enzymes commonly used in industry. This study describes the production of lipolytic enzyme *via* a newly isolated strain of *Aspergillus oryzae* under a submerged fermentation process. Five strains of *A. oryzae* were isolated from oil-contaminated soil and water as well as dead decaying organic matter. Qualitative screening revealed that *A. oryzae* RBM4 strain was a lipase producer, and the process was optimized for enhanced production. Incubation time, incubation temperature, initial pH, use of agricultural by-products, nitrogen sources, and substrates were tested. The results revealed that initial pH 5.5(12.7 U/mL/min) in 72 h (19.39 U/mL/min) at 30 °C (27.40 U/mL/min) sorghum (35.66 U/mL/min), $NaNO_3$ (17% more than blank), yeast extract (47.95 U/mL/min), and Shan ghee (58.12 U/mL/min) were the best conditions. Extracellular lipase production was increased up to 78% by applying all the above conditions.

Keywords: Lipase; Physico-chemical parameters; Fermentation; Running head; Submerged fermentation; Lipolytic Aspergillus oryzae

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INTRODUCTION

Enzymes are useful biocatalysts for various industrial processes and chemical reactions under mild conditions with high selectivity. Moreover, enzymes also have various applications in the environmental and biotechnology fields at commercial levels. Lipases have broad-spectrum usage in these fields (Kumar *et al*. 2017). Advances in biotechnology are providing more chances for the development of enzymes to meet the demands. Among hydrolytic lipases, the triacylglycerol acyl hydrolases (EC 3.1.1.3) are characterized by their unique ability to emulsify the substrate and to hydrolyze glycerides to free fatty acids and glycerols. Due to these unique characters, this enzyme has wide applications in number of procedures for the synthesis of variety of dairy products, fats and oils, and it has applications in detergents, cosmetics, and medicine (Priji *et al*. 2016). Lipases are widely used in bakery products and organic synthesis to improve the flavor and aroma of yogurt, cheese, butter, and milk (Iftikhar *et al*. 2011). Lipases are extensively found in nature, but only microbial lipases are commercially important (Vishwe *et al*. 2015). Various microorganisms produce lipases including numerous fungi and bacteria. *A. niger*, *A. oryzae*, and *R. oligosporous* showed high productivity of lipases (Vishwe *et al*. 2015). As lipases are in great demand at industrial levels, a cost effective synthesis involving low-cost culture media under submerged fermentation would be optimal (Grazia and Trono 2015).

Maia *et al.* (1999) reported a similar optimum growth temperature for the *Fusarium* and *Aspergillus* spp., respectively. Helal *et al*. (2017) and Babic *et al*. (2017) evaluated parameters for lipase production from fungus by using one factor at a time and selected the optimum conditions for lipase production by *Rhizopus oryzae* R1 and reported highest activity at 30 °C. This finding indicates the sensitivity of lipase activity coupled with less fungal growth or inactive nature of the enzyme itself (Iftikhar *et al*. 2003; Zainab *et al.* 2017). According to Amin and Bhatti (2014), high temperature affects the cell membrane composition as well as stimulates protein catabolism, leading to cell death.

Cellulosic materials are attractive for low-cost production. Agricultural byproducts such as bran from wheat and rice, corncobs, mustard oil, bagasse, *etc.*, are used to provide the required nutrients for enzyme production and are renewable cheap resources having carbon, nitrogen, and mineral sources (Salihu *et al*. 2012). Submerged fermentation is advantageous over solid-state due to multiple choices of microorganisms capable of growth under an abundant supply of moisture. Moreover, controlling and monitoring of parameters such as temperature, initial pH, humidity, and air flow don't have any regulation restriction, and solid-state fermentation has a narrow range w.r.t. these parameters (Sutto-Ortiz *et al*. 2017). Microbial lipase production is greatly influenced by the initial pH, moisture content, amount of substrate, inoculum size, incubation time and temperature, composition of production medium, carbon sources, organic and inorganic nutrients, and dissolved oxygen concentration (Amin and Bhatti 2014).

As compared to fauna and flora, microorganisms can produce high yields of lipases through bioprocess technology. This production can be used in industries such as food, oil and fat, detergent, pulp and paper, leather and textile, along with in-organic synthesis, cosmetics, and biodiesel production (Berhanu and Amare 2012). Microorganisms have better stability, selectivity, and substrate specificity which add to its usefulness. Research has focused on its process optimization, modeling, and design for its huge production and industrial implementation (Sarmah *et al*. 2018).

Previously solid state fermentation has been done for the production of lipase from fungi but in this study, various physico-chemical parameters were studied to enhance lipase production by submerged fermentation by *A. oryzae* on rice straw, wheat bran, rice bran, wheat straw, corncobs, and sorghum as substrates for the first time (Arun *et al.* 2017). This fungal strain is an extensively investigated lipase-production microorganism. It is on the Generally Recognized As Safe (GRAS) list of the Food and Drug Administration (FDA) in the United States, since it produces extracellular lipases and facilitates the extraction from fermentation media (Zhou *et al*. 2012).

EXPERIMENTAL

Materials

Lipase-rich samples were collected from various lipids/oil rich habitats including oil-contaminated soil, dead-decaying organic matter (such as oily bread, cooked turnip, cooked beans *etc.*), and oil-contaminated water.

Identification of lipolytic fungi

The Microbial Identification Method was not performed since already identified strains of a filamentous fungus *Aspergillus oryzae* CJLU-31 of *Aspergillus* sp were used for lipase production.

A serial dilution technique was used for production of fungi from its glycerol stocks as reported (Akano and Atanda 1990). Agar plates with polyoxyethylene sorbitan monooleate (Tween-80) were prepared to isolate the lipolytic fungi. These plates were incubated at 25 ± 2 °C and examined regularly after 24 h. The appearance of a visible precipitate, resulting from the deposition of calcium salt crystals formed from the fatty acids liberated by the lipase, indicated lipolytic activity. Individual colonies of isolated lipolytic strains were stored on 4.0% potato dextrose agar (PDA) medium.

Fermentation technique and lipase assay

Submerged fermentation was used for lipase production. Approximately 250 mg of wheat bran was added to 25 mL E-fermentation medium, where the broth contains 2 g peptone and glucose, 0.5 g KH_2PO_4, 0.1 g $(NH_4)_2SO_4$, 0.1 g $(NH_4)_2CO_3$, 0.1 g $MgSO_4$, 1.0 mL olive oil, and dissolved in 100 mL of distilled water. The P-medium (production medium in which broth contains 5 g peptone, 3 g yeast extract, 2 g of beef extract, and 5 mL of lipid substrates olive oil, and dissolved in 100 mL of distilled water). The Initial pH was adjusted to 7.2 by means of pH meter and autoclaved at 37 °C. After sterilization, under aseptic conditions, the sterilized flasks were inoculated with freshly prepared mycelia of *A. oryzae* and incubated at 30 ± 2 °C for two days.

The lipase activity {U(µmol/min)/mL/min} was estimated by the Kundu and Pal (1970) method, in which 1.0 mL enzyme extract was mixed with substrate containing 10% (w/v) gum acacia and olive oil having 2.0 mL of 0.6% (w/v) $CaCl_2$. H_2O in phosphate buffer (Initial pH 7.0). The reaction was carried out at 30 ± 2 °C for 10 min. The reaction was stopped by adding 10 mL acetone/ethanol (1:1 ratio) and then titrated against 0.05 M NaOH to measure the liberated fatty acids (without enzyme and without substrates controls, respectively).

Protein estimation

Whole protein content was measured (Bradford 1976). Approximately 0.2 mL of the extract was added to the reagent in 5 mm test tubes and vortexed. The solution was measured at 595 nm wavelength on a spectrophotometer (CECIL EC-7200 Series, Edinburgh, Scotland, UK). The blank was prepared by adding 0.2 mL of distilled water instead of enzyme extract. Bovine serum albumin was used to make the standard curve for the total protein calculation.

Statistics

All parameters were compared by the protected LSD after Snedecor and Cochran (1980) method by using the Costat computer software (Lahore, Pakistan) in MS Excel 2010.

RESULTS AND DISCUSSION

Lipases are commonly used microbial enzymes with numerous applications in fermentation and biochemical processes (Kiran *et al*. 2001). In this study, the physio-chemical parameters for obtaining the highest yield of lipase were optimized.

The initial pH can influence microbial growth and ultimately the product formation due to its effect on the solubility of nutrients, ionization of the substrate, and its availability to the microorganisms. Filamentous fungi are able to flourish over a wide Initial pH range

in submerged fermentation conditions (Shaheen *et al*. 2008). The effect of initial pH (3.5 to 8.5) on lipase production by *A. oryzae* RBM[4] was investigated (Fig. 1).

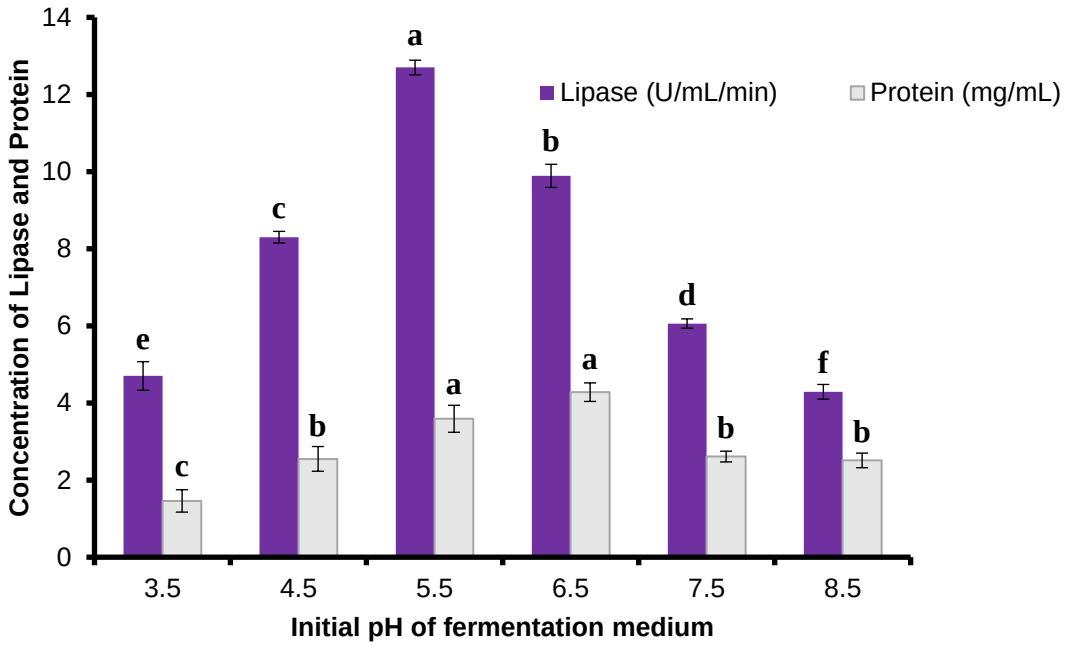

Fig. 1. Amount of lipase and protein at different initial pH of fermentation medium; a = maximum amount of lipase and protein after applying statistical tool; b,c,d,e = decreasing amounts of amount of lipase and protein after applying statistical tool; and f = least amount of lipase and protein after applying statistical tool

The highest enzyme activity (12.7 ± 0.19 U/mL/min) was seen at initial pH 5.5, while the content of protein at this initial pH was 3.59 ± 0.37 mg/mL. Lipase activity fluctuated with changes in Initial pH, as the high activity is previously seen in the acidic medium (Shimaa *et al*. 2017). However the enzyme activity dropped to (10.1± 0.25 U/mL/min), but the concentration of protein at this initial pH was increased to 4.8± 0.57 mg/mL. Each microorganism has a unique optimal Initial pH for its development and activity. The present study shows results similar to Amin *et al*. (2014), who reported that a higher amount of lipase was produced (373.9 U/gds) at the same initial pH by *A. melleus*.

Incubation time is important in the metabolism of an organism. The rate of lipase production by *A. oryzae* RBM[4] was studied (Fig. 2). Lipase activity in fermented broth increased progressively with the incubation period to reach a maximum (19.39 ± 0.57 U/mL/min) at 72 h. Further increases in the incubation period decreased the lipase production. Therefore, 72 h was the optimal incubation time for *A. oryzae* RBM[4], yielding 34% increased lipase productivity. For this incubation period, the total protein content was 4.33 ± 0.79 mg/mL. Eugenia *et al*. (2016) reported that prolonged incubation reduced the lipase yield, which is due to inactivation of the desired enzyme. This effect might be due to the adaptation of microorganism to the environment. Savetlana *et al*. (2005) described that lipase activity is high in the exponential phase and reduced at the late logarithm phase due to the production of $C_6H_8O_7$.

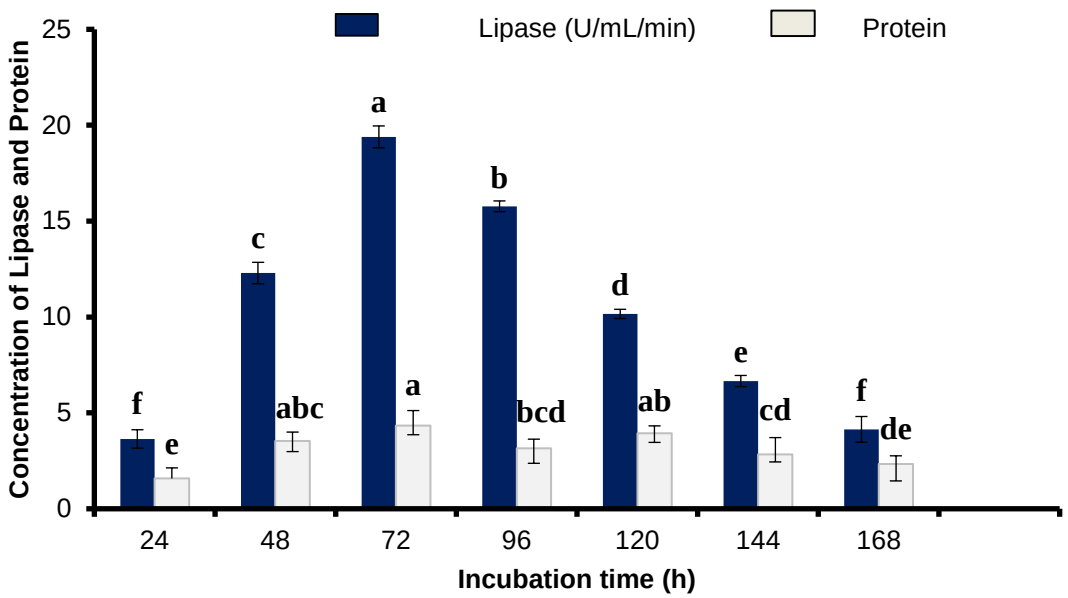

Fig. 2. Incubation time and lipase production; a = maximum amount of lipase and protein after applying statistical tool; b,c,d,e= decreasing amounts of amount of lipase and protein after applying statistical tool; and f= least amount of lipase and protein after applying statistical tool

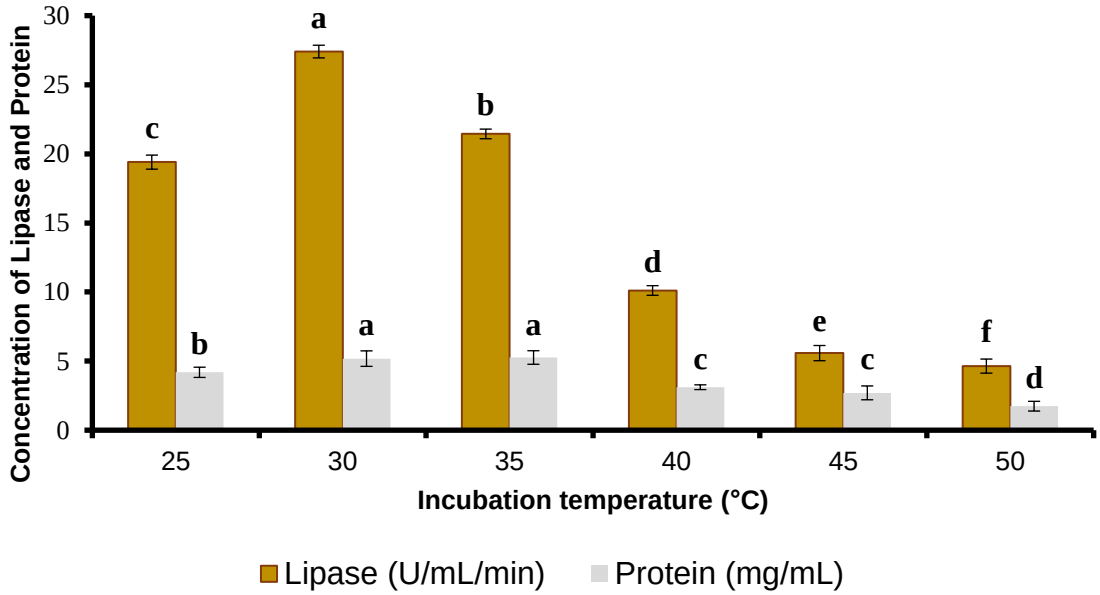

Fig. 3. Amount of lipase and Protein formed at various incubation temperatures: a = maximum amount of lipase and protein after applying statistical tool; b,c,d,e = decreasing amounts of amount of lipase and protein after applying statistical tool; and f= least amount of lipase and protein after applying statistical tool.

A. oryzae RBM[4] produced the maximum amount of lipase (27.40 ± 0.46 U/mL/min) at 30 °C, with 5.18 ± 0.56[a] mg/mL of total protein. However, below and above this temperature, *A. oryzae* RBM[4] gave a decreased rate of lipase production. The total protein content obtained was maximum (5.26 ± 0.49 mg/mL) at 30 °C. The rate of production for both the enzyme concentration and the amount of protein was seen decreased with the increase of temperature.

The production of hydrolytic enzymes for commercial uses should be cheap, with reduced energy requirements, low capital investment, low waste water output, high concentration of metabolites production, and low down streaming processing cost. The substrate specificity was determined with agricultural by products (wheat bran, rice bran, wheat straw, rice straw, corncobs, and sorghum) in 1.0 % concentration (Fig. 4).

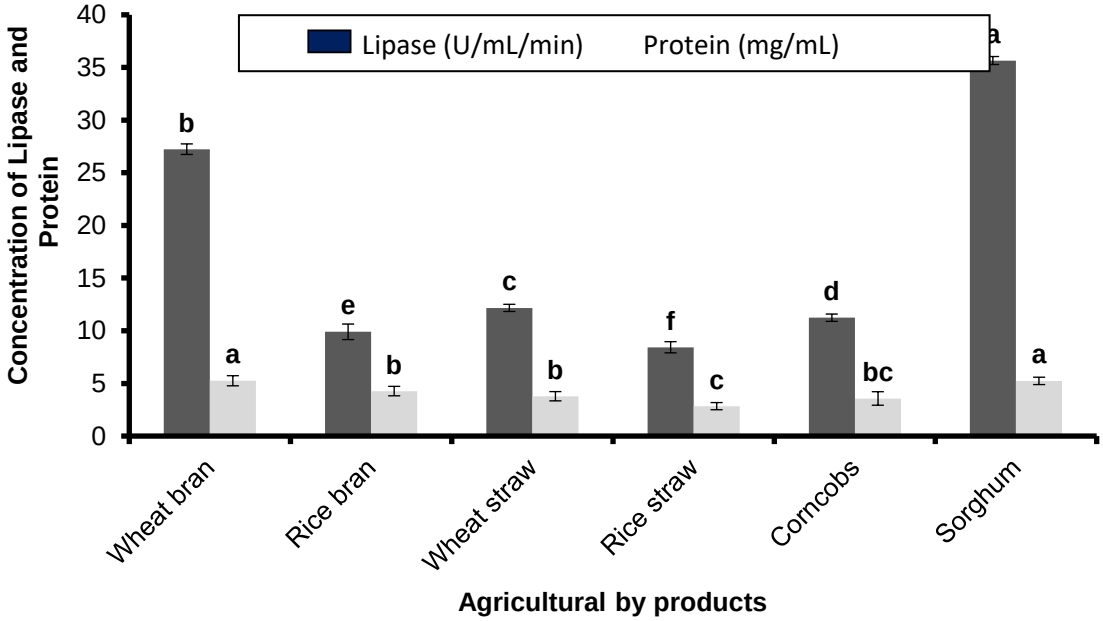

Fig. 4. Agricultural by-products and amount of lipase produced; a = maximum amount of lipase and protein after applying statistical tool; b, c, d, e = decreasing amounts of amount of lipase and protein after applying statistical tool; and f= least amount of lipase and protein after applying statistical tool.

The highest enzyme activity of 35.66 ± 0.38 U/mL/min with total protein content of 5.24 ± 0.35 mg/mL was obtained when sorghum 250 mg was used. However, a higher amount of total protein (5.25 ± 0.48 mg/mL) was obtained with wheat bran. A 23.0% increase in lipase production was obtained upon using sorghum as the substrate carrying the highest concentration of fats (Nambiar *et al*. 2011).

The formation of primary and secondary metabolites by microorganisms is highly influenced by their growth, which depends on the nutrients provided. Nitrogen content is one of the most important factors. The influence of inorganic substances on the activity of *A. oryzae* RBM[4] was investigated by supplementing the growth media with 0.15% $NaNO_3$, KNO_3, and $Ca(NO_3)_2$ (Fig. 5).

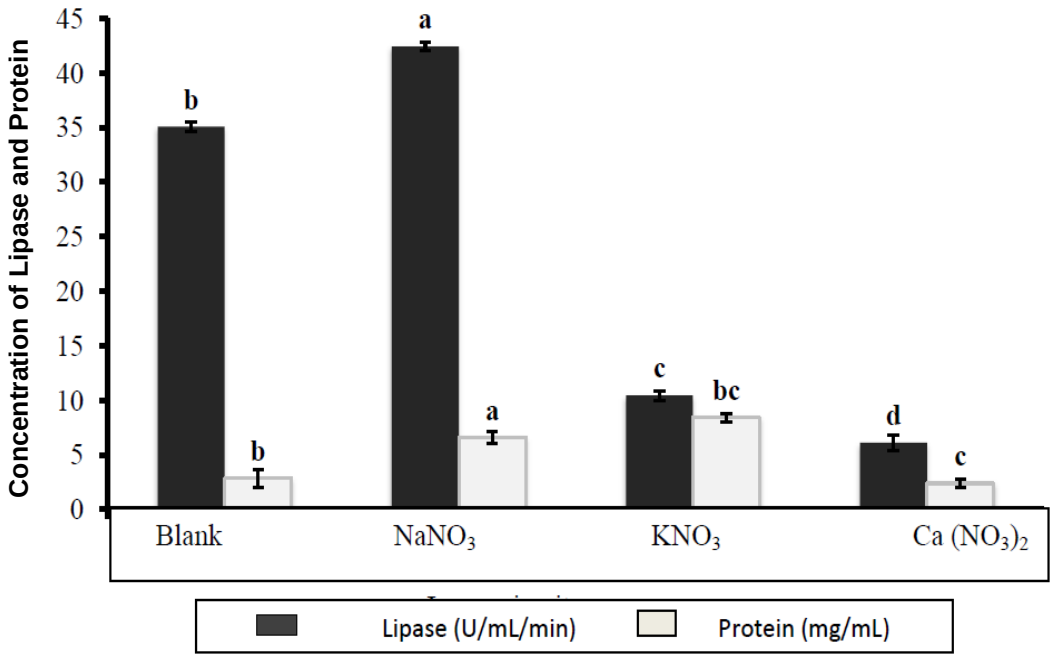

Fig. 5. Inorganic nitrogen sources and lipase production; a = maximum amount of lipase and protein after applying statistical tool; b, c, d, e = decreasing amounts of amount of lipase and protein after applying statistical tool; and f = least amount of lipase and protein after applying statistical tool

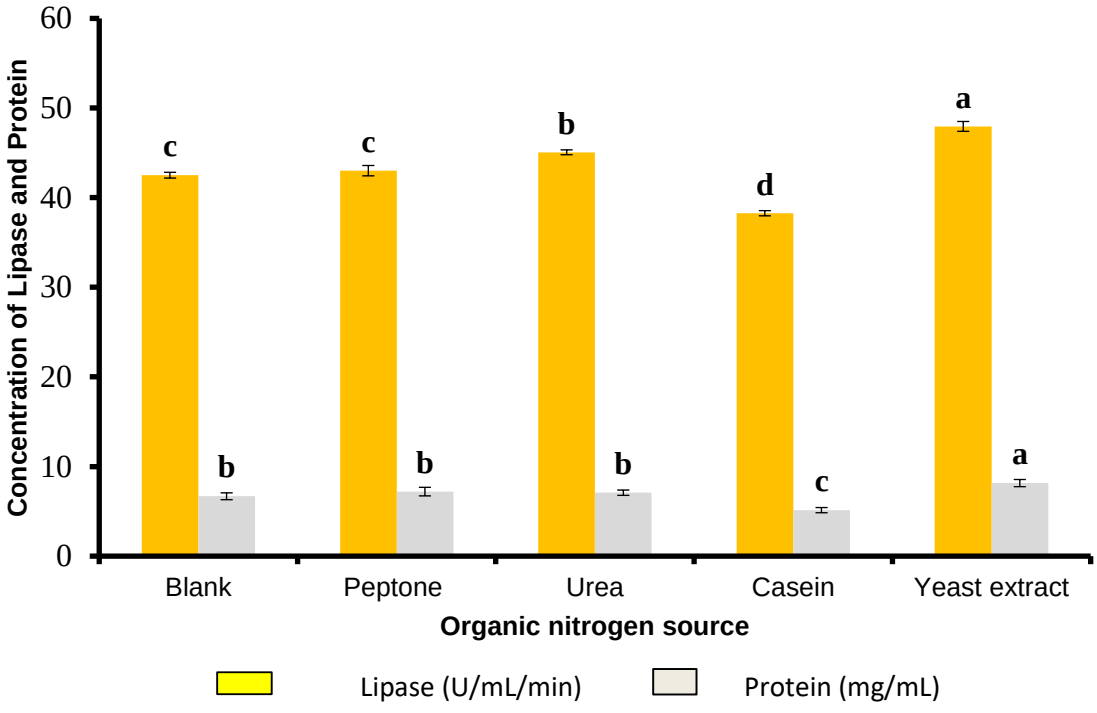

Fig. 6. Effect of organic nitrogen sources on lipase amount produced; a = maximum amount of lipase and protein after applying statistical tool; b, c, d, e = decreasing amounts of amount of lipase and protein after applying statistical tool; and f = least amount of lipase and protein after applying statistical tool

The $Ca(NO_3)_2$ yielded the minimum lipase production (6.09 ± 0.70 U/mL/min). However, maximum enzyme activity (42.42 ± 0.39 U/mL/min) was obtained when the fermentation media was supplied with 0.15 % (w/v) $NaNO_3$ solution along with maximum total protein (6.17 ± 0.57 mg/mL) content. Likewise, the effect of organic nitrogen sources (peptone, urea, casein, and yeast extract) upon lipase activity was investigated (Fig. 6).

A. oryzae RBM[4] gave higher lipase production (47.95 ± 0.55 U/mL/min) and the maximum total protein content (8.15 ± 0.40 mg/mL) on addition of 0.1%(w/v) yeast extract. Organic nitrogen sources had more positive effects on enhancing the lipase production than those of inorganic sources, wherein $NaNO_3$ and yeast extract were the optimum substrates. The effect of organic nitrogen sources upon lipolytic activity has been reported previously. According to Imandi *et al*. (2010), urea (organic nitrogen source) showed higher lipolytic activity, whereas Ramani *et al*. (2010) found an inhibitory effect upon lipase production. Numerous research studies have been conducted on the effect of a nitrogen source for the synthesis of enzymes. The increase in lipase activity upon using yeast extract in this study is similar to results in Oshoma *et al*. (2010). To examine the suitability of a particular substrate on the growth and development of microorganisms, various oil and fat residues (Shan ghee, Shan oil, Daldaghee, Dalda oil, Canola oil, and Olive oil) were examined for lipase production by *A. oryzae* RBM[4] (Fig. 7).

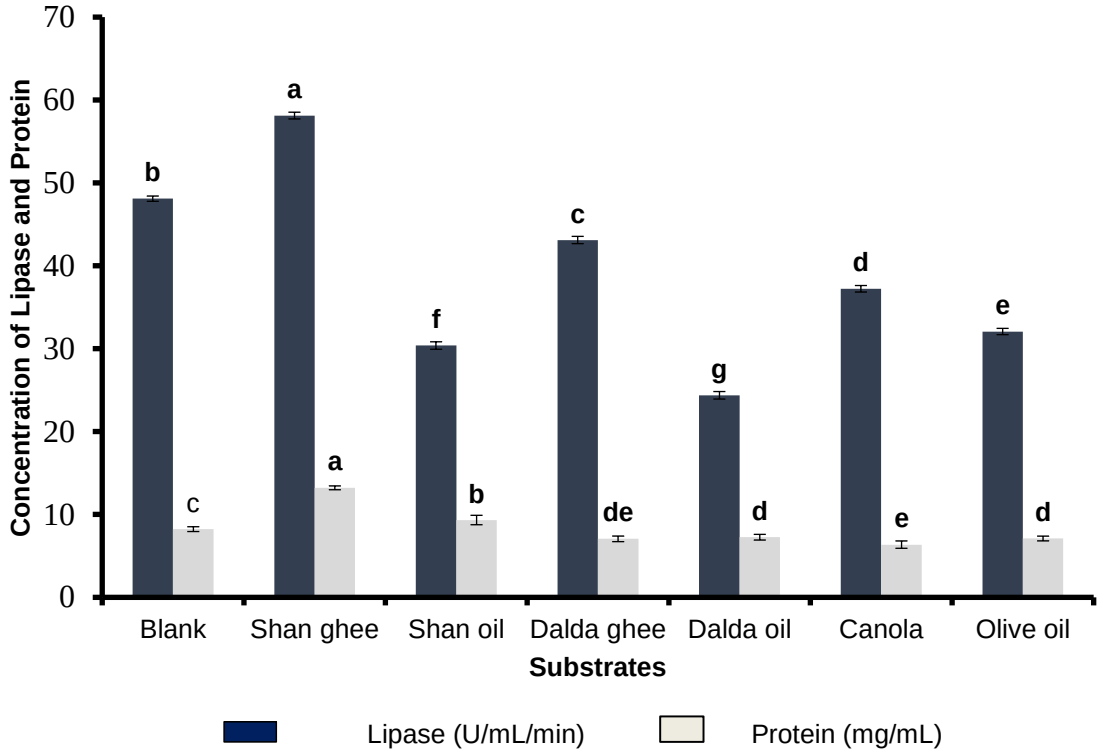

Fig. 7. Effect of various oil substrates on the production of lipase; a = maximum amount of lipase and protein after applying statistical tool; b, c, d, e = decreasing amounts of amount of lipase and protein after applying statistical tool; and f = least amount of lipase and protein after applying statistical tool

A. oryzae RBM[4] produced the highest quantity of lipase (58.12 ± 0.41 U/mL/min) and total protein (13.21 ± 0.23 mg/mL) when shan ghee (0.75%) was used as the substrate. Toscano *et al*. (2013) demonstrated that the wheat bran with olive oil and castor oil cake used as a substrate augmented the activity of enzyme.

CONCLUSIONS

1. *A. oryzae* has great potential for extracellular lipase production at initial pH of 5.5 by incubating shan ghee as substrate, sorghum as agriculture by product, $NaNO_3$ and yeast extract as inorganic and organic nitrogen sources, respectively, for 72 hours at 30 ^oC. This potential can be further enhanced several times through the use of above physical parameters at different conditions.
2. In the future, higher lipase production can also be achieved by modifying the above physical parameters and by using various substrates as inducible agents and other identified strains of fungi (other *Aspergillus* sp. and other than *Aspergillus* sp.). Moreover, new strains of filamentous or non- filamentous fungi can also be isolated, screened and identified from natural sources (soil, water, land, plants or animals) and not only fungal strains but bacterial strains can also be used as microbial source to check for their lipase activity

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