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Production, Partial Purification and Characterization of Lipase from *Aspergillus flavus* KUF108

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Abstract. Fungi isolated from oil contaminated soils were screened for exogenous lipolytic activity. Optimization of fermentation conditions such as substrate, temperature, pH, moisture content, incubation period, carbon source, nitrogen source and metal ions for maximum lipase production was examined under solid state fermentation by the local isolate of *Aspergillus flavus* KUF108. Purification of crude enzyme was carried out by ammonium sulphate precipitation, dialysis and DEAE cellulose column chromatography. The lipase was found to be active at pH 5 and 50 °C, and stable between pH 5-6 and 40-60 °C. The apparent molecular weight of purified enzyme was 44 kDa.

Keywords: *Aspergillus flavus*, lipase, solid state fermentation, agro-industrial waste, enzymatic characterisation

Introduction

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in food, dairy, detergent and pharmaceutical industries (Hassan *et al*., 2009). Lipases are by and large produced from microbes and specifically fungal lipases play vital role in commercial ventures (Gupta *et al.*, 2004). Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995). The lipase can be produced either by solidstate or submerged fermentation by involving microorganisms such as bacteria, fungi and yeast (Shu *et al.*, 2006; Sharma *et al*., 2001). Lipases from filamentous fungi are preferably used for industrial applications due to the feasibility of obtaining them in high concentrations by solid-state fermentation process (Mala *et al*., 2007; Mahadik *et al.*, 2002). For developing industrial fermentation, designing media and optimizing fermentation conditions are of critical importance because these factors could strongly interfere with the yield of lipase production (Rajendran and Thangavelu, 2009).

Solid state fermentation may produce industrial enzymes at lower cost due to the possibility of using agro-industrial residues as culture media. Solid wastes from the production of vegetable oils have been widely used for the production of industrial enzymes, antibiotics, biopesticides, vitamins and other biochemicals because they are the excellent support for the microbial growth as well as interesting sources of nutrients, requiring low or no supplementation (Castilho *et al.*, 2000). Solid state fermentation has many advantages over submerged fermentation including economy of space needed for fermentation, non-requirement of complex machinery equipment and control system, less energy consumption, lower capital and recurring expenditure etc. (Satyanarayana, 1994). Solid state fermentation holds tremendous potential for the production of enzymes (Sathya *et al.*, 2009). In the present study, effect of various industrial wastes on lipase production by the local isolate of *Aspergillus flavus* KUF108 was determined using solidstate fermentation process.

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Materials and Methods

Microorganism. Lipase producing strain of *Aspergillus flavus* KUF108 was isolated from oil-spilled soil sample, near coconut oil extracting industry, Kerala, India (Palaniswamy *et al*., 2008). Isolated fungi were identified on the basis of cultural and morphological features (Gilman, 1971). The fungal strain has been deposited at Karpagam Microbial Culture Collection Centre (KMCCC), Coimbatore, India.

Solid state fermentation. Ten grams of agro-industrial waste (rice bran, wheat bran, coconut oil cake, gingelly oil cake, cotton seed cake and groundnut oil cake) was taken in 250 mL Erlenmeyer flasks, moistened with 10 mL of sterile distilled water in the ratio of 1:1 w/v and sterilized. After coo-ling, the flasks were inoculated with 1 mL of spore suspension (10^6 spores/ mL) and the contents were mixed and incubated at 30 °C for seven days. Enzyme extraction was performed by adding 100 mL of distilled water to the solid

moulding medium and shaking the mixture in a rotary shaker (100 rpm) for 1 h. The extracts were squeezed through a muslin cloth and clarified by centrifugation at 10,000 × g for 5 min (Sathya *et al*., 2009). The supernatants were used as crude enzyme.

Enzyme assay. According to Hasan *et al*. (2009), use of olive oil as substrate for titrimetric method has been reported by many scientists (Kashmiri *et al.*, 2006; Fuji *et al*., 2003; Pignede *et al.*, 2000; Macedo *et al*., 1997).

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis (Wantanabe *et al.*, 1977). One mL of the culture supernatant was added to the reaction mixture containing 1 mL of 0.1 M tris-HCl buffer (pH 8.0), 2.5 mL of deionised water and 3 mL of olive oil. The reaction mixture was blended well and incubated at 37 °C for 30 min. Both test and blank samples were assayed. Immediately after starting the incubation, 1 mL of the culture supernatant was pipetted into a 50 mL Erlenmeyer flask marked blank and stored at 4 °C. After 30 min, the test solution was transferred to a 50 mL Erlenmeyer flask. 3 mL of 95% ethanol was added to stop the reaction. Liberated fatty acid was titrated against 0.1 M NaOH using thymolphthalein blue as indicator. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per minute under specified assay conditions. Enzyme activity was expressed as units per gram of dry substrate.

Protein assay. Protein content of the supernatant was quantified by the method of Lowry *et al*. (1951) with bovine serum albumin as standard and was expressed as mg/mL.

Optimization studies. Using different agro-industrial waste materials (wheat bran, rice bran, cottonseed oil cake, coconut oil cake and groundnut oil cake), lipase production was studied at different pH (4-8), temperature (30-70 °C), moisture content (10-50%) and incubation period (3-7 days).

Different carbon sources (lactose, maltose, mannitol, starch and sucrose), nitrogen sources (peptone, yeast extract, casein, urea and albumin) and metal ions (magnesium sulphate, calcium chloride, sodium chloride, ferrous sulphate and zinc chloride) were supplemented separately to a final concentration of 1% (w/v) in solid media. After fermentation, the lipase activity was estimated.

Partial purification for enzyme characterization. *Ammonium sulphate fractionation*. Solid ammonium sulphate was added to the crude enzyme to 40-80% saturation. The precpitate was collected by centrifugation, dissolved in minimal volume of 0.2 M phosphate buffer (pH 6.2) and dialysed overnight against the same buffer at 4 °C. (Beisson *et al*., 2000).

DEAE-cellulose chromatography. The enzyme solution obtained in the above step was applied to DEAE-cellulose column (2.4 × 45 cm), pre-equilibrated with 5 mM phosphate buffer (pH 6). The enzyme was eluted with the same buffer at a flow rate of 10 mL/h (Giraud *et al*., 1993).

Molecular weight determination. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% (w/v) acrylamide slab gel with 25 mM Tris/192 mM glycine buffer (pH 8) that contained 0.1% (w/v) SDS as the running buffer, (Laemmli, 1970).

Enzyme characterization. *Determination of optima and stability of temperature and pH.* Optimum temperature for activity of the lipase was determined at selected temperatures from 30 to 70 °C. In each case, the substrate was pre-incubated at the required temperature before the addition of enzyme. Optimum pH was determined by monitoring lipase activity at pH 4-8.

Optimum substrate concentration. Optimum substrate concentration for maximum enzyme activity was determined in terms of maximum retention velocity (V_{max}) and Michaelis constant (K_m) at which the retention velocity is half maximum. For this, various concentrations of protein in 0.05 M sodium acetate buffer were incubated with purified enzyme preparation. The accurate values of V_{max} and K_m were obtained from the Lineweaver-Burk plot (Lineweaver and Burk, 1934) and Eadie- Hofstee plot.

Results and Discussion

Optimization of cultural parameters. *Influence of substrate on enzyme production.* Selection of ideal industrial waste for enzyme production in a solid state fermentation process depends upon several factors, mainly related to the cost and the availability of the substrate material and thus may involve screening of several agro-industrial residues (Pandey *et al.*, 1999). In the light of several advantages of SSF, lipase production from *A. flavus* KUF108 was attempted with various industrial waste substrates. The result in the present study indicated that lipase enzyme production pattern varied with agro-industrial residues.

Various substrates like (rice bran, wheat bran, coconut oil cake, gingelly oil cake, cotton seed cake and groundnut oil cake) were screened for lipase production. *A. flavus* showed maximum enzyme yield of 53.33 U/g of dry substrate after 72 h of incubation on wheat bran (Fig.1). Di Luccio *et al.* (2004) observed a similar value of lipase activity (21 U/g) after 48 h of fermentation with the strain of *P. simplicissimum* grown on soy cake. Mala *et al*. (2007) reported maximum lipase

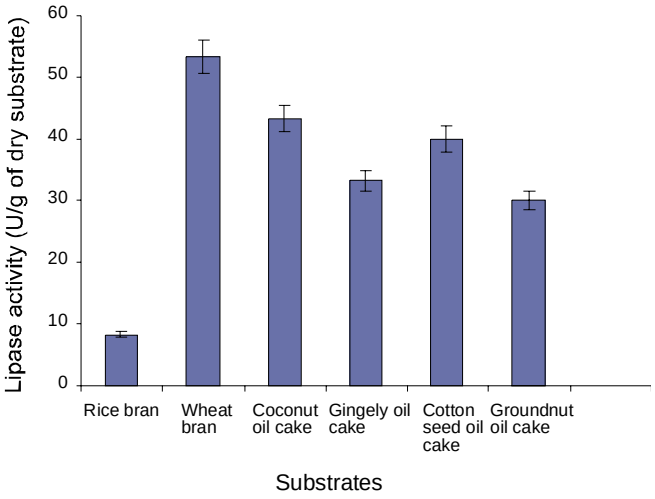

Fig. 1. Influence of various substrates for lipase production.

production by *A. niger* to be 271.6 U/g dry sustrate (ds) using wheat bran as the substrate.

Influence of temperature and pH on enzyme production. Lipase production at different temperatures (30-70 °C) was examined for 72 h keeping other fermentation conditions constant. Lipase production increased with increase in temperature from 30 to 50 °C. Maximum production of lipase (40 U/g of dry substrate) was obtained at 50 °C and production declined at 60 °C. Pau and Omar (2004) reported that *A. flavus* USM A10 showed maximum lipase activity of 5.9±0.03 U/g substrate within the temperature range of 28 to 30 °C. Higher temperature resulted in lower lipase activity which may be related to the low enzyme stability at higher temperature. Although many other enzymes produced by bacteria and yeast show maximum activities at high temperatures, such as *Pseudomonas aeruginosa* (70 ºC) (Karadzic *et al*., 2006) and yeast *Kurtzmanomyces* sp. (75 ºC) (Kakugawa *et al*., 2002), just few fungal lipases reported in literature present such thermophilic behaviour (Gutarra *et al*., 2005).

Lipase production by *A. flavus* was observed at different pH in the range of 4-8. Growth and lipase production ceased at pH 8. Maximum lipase production of 22.5 U/g of dry substrate was observed at pH 5. Optimum pH for the production of lipase was 6.0 by *A. niger* (Seitz, 1974), 6.25 for *A. oryzae* (Seitz, 1974) and 7.0 for *Candida deformans* (Murderhwa *et al*., 1985).

Influence of moisture content and incubation period. Water is present in very limited amount in SSF system and thus an optimum content is important as it determines the productivity of a SSF process (Lonsane *et al*., 1985). The effect of moisture content was examined in the range of 10 to 50% for the analysis of lipase production. *A. flavus* showed maximum enzyme production at 30% with 18.33 U/g of dry substrate. Zadrazil and Brunnert *et al.* (1981) have indicated that a low moisture level leads to swelling which might decrease enzyme production. A moisture content of 1:1 (substrate :water, w/v) was optimal for lipase production by *Candida rugosa* (Rao *et al.*, 1993).

Lipase enzyme production by *A. flavus* was examined on various incubation days ranging from day 3 to 7. *A. flavus* showed the maximum of 40 U/g of dry substrate enzyme production on day 4. Cho *et al.* (2007) observed maximum lipase activity (40 U/mL) for *Penicillium chrysogenum* at 20 ºC on the fifth day. Mohawed *et al.* (1988) reported peak lipase productivity by *A. fumigatus* after 5 days of incubation. *A. fumigatus* and *A. nidulans* exhibited peak lipase production after 10 days of incubation (Ogundero, 1982).

Influence of carbon, nitrogen and metal ions. Several carbon sources like glucose, maltose, sucrose, lactose and mannitol were assayed for the enzyme production. *A. flavus* showed maximum enzyme production with mannitol (36.66 U/g of dry substrate). Fadiloglu and Erkmen (2002) reported enhanced lipase activity (2.03 U/mL) in the media enriched with glucose for *Candida rugosa*.

Several nitrogen sources like urea, albumin, peptone, casein and yeast extract were also assayed for the enzyme production. *A. flavus* showed maximum enzyme production with peptone (31.67 U/g of dry substrate). Organic nitrogen sources were found to increase lipase synthesis by *Candida rugosa* in the presence of olive oil. Supplementation of nitrogen sources resulted in increasing growth in the range of 6.75 U/g for *A. flavus* (Pau and Omar, 2004). Several metal ions like $ZnCl_2$, $FeSO_4$, $MgSO_4$, NaCl and $CaCl_2$ were assayed for the enzyme production. *A. flavus* showed maximum enzyme production with $MgSO_4$ (33.33 U/g of dry substrate).

Partial purification and enzyme characterisation. Purification of lipase resulted in two fold purification with 72% recovery by ammonium sulphate precipitation. Purification of crude enzyme through DEAE cellulose column chromatography gave purification fold of 58.09 with 43.39% recovery of lipase from *A. flavus* (Table 1).

The molecular weight of lipase enzyme produced by *A. flavus* was found to be 44 kDa by SDS-PAGE. Dubois *et al.* (1956) reported mass of protein in *A. niger* in the range of 35-40 kDa (Fig. 2).

Enzyme characterisation. *Temperature and pH stability.* Temperature is a critical factor for maximum enzyme activity

Table 1. Purification of lipase enzyme from *Aspergillus flavus*

Fractions	Total volume (ml)	Enzyme activity (U/g)	Protein (mg/mL)	Total protein (mg)	Total activity (U/g)	Specific activity (U/g)	Purification (Fold)	Recovery (%)
Culture filtrate	100	58.3	13.29	1329	5330	4.01	1	100
Ammonium sulphate precipitation	50	76.6	9.24	462	3830	8.29	2.07	71.88
Dialysis	25	98.3	7.48	187	2457.5	13.14	32.76	64.13
DEAE cellulose column chromatography	10	106.6	2.19	21.9	1066	48.6	58.09	43.39

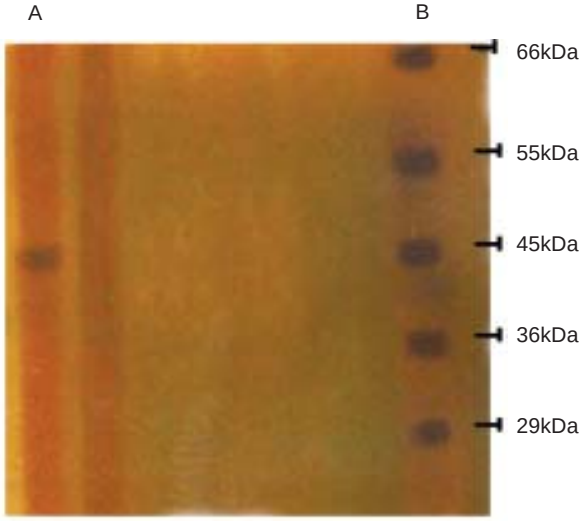

Fig. 2. Molecular weight of lipase produced by *A. niger*.

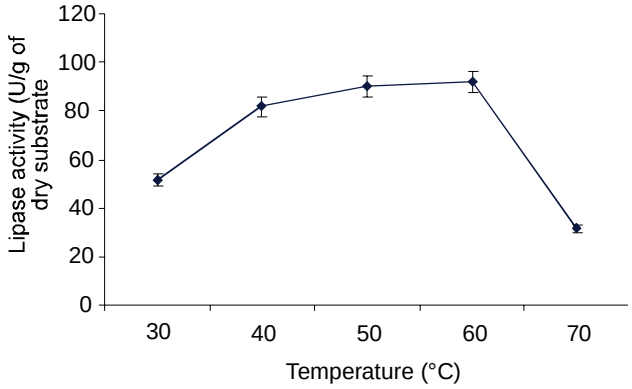

Fig. 3. Effect of various temperatures on lipolytic activity.

and it is a prerequisite for industrial enzymes to be active and stable at higher temperatures. The residual activity was measured to determine lipase stability after one hour incubation at different temperatures ranging from 30-70 °C. Maximum stability of the enzyme was observed in the temperature range of 40 to 60 °C, when the enzyme was incubated for 1 h (Fig. 3). So the thermostability of the enzyme was found to be up to 60 °C for *A. flavus.*

Almost all lipases produced by fungi reported in literature have low thermal stability, being unstable at temperatures above 40 °C. In contrast, the lipase produced through SSF showed good thermal stability, more than that of the lipase produced by submerged fermentation by *Penicillium* spp. (stable at 50 °C for 15 min) (Sztajer *et al*., 1992) and also higher than that of the lipase produced in SSF by *Rhizopus homothallicus* (Mateos Diaz *et al.*, 2006).

The lipase of *P. aurantiogriseum*, despite having maximum activity at 60 °C, showed low thermal stability at temperatures higher than 28 °C, presenting a residual activity of only 32% after 30 min of incubation at 50 °C (Lima *et al*., 2004). Lipase of *Penicillium wortmanii*, is described as a moderately thermostable enzyme, but it retains only 55% of the initial activity after 1 h incubation at 50 °C (Costa and Peralta, 1999). Similar thermal stability as that of this crude enzyme has been reported for the lipase of the halophilic archea *Natronococcus* sp., which presented more than 90% of initial activity after 1 h incubation at 50 °C (Boutaiba *et al.*, 2006).

The effect of pH on the activity of lipase was studied at various pH from 4-8. The optimum pH for lipase enzyme from *A. flavus* was determined as 5 (Fig. 4). The pH stability curve showed that the lipase was stable at pH 5-6. Most microbial lipases are stable in the pH range of 2 to 10.5 as reported by many researchers. Similar results have been reported for other fungal lipases (Falony *et al.*, 2006, Salleh *et al*., 1996).

Substrate concentration. V_{max} and K_m values for lipase of *A. flavus* were determined from Line Weaver and Eadie-Hofstee plots. The reaction between the substrate and the enzyme was carried out using initial concentration of the substrate to determine the kinetic constants V_{max} and K_m (Table 2).

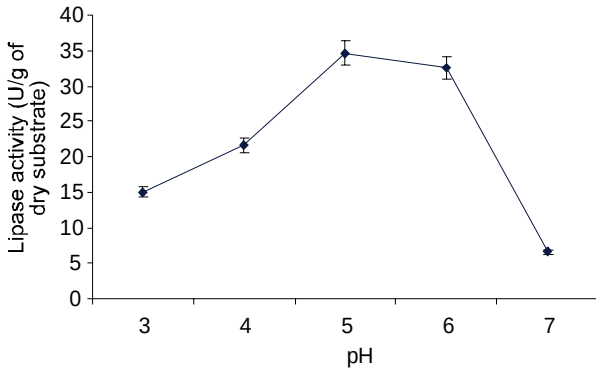

Fig. 4. Effect of various pH on lipolytic activity.

Table 2. Properties of lipase of *Aspergillus flavus*

Properties	
Optimum pH	5
Optimum temperature(ºC)	50
V_{max} (U/g)	85
K_m (mg/mL)	0.8
Molecular weight (kDa)	44

The kinetic parameters of purified lipase enzyme from *A. flavus* were determined and the V_{max} and K_m value were found to be 85 U/mg and 0.8 mg/mL, respectively. A low K_m value represents a high affinity. The K_m values of the enzyme range widely, but for the most industrially relevant enzymes, K_m has been reported to be in the range of 10^{-1} and 10^{-5} M (Fullbrook, 1996). The K_m and V_{max} values were 0.7 mg/mL and $0.97x10^3$ U/min, respectively (Pabai *et al*., 1995).

Conclusion

A. flavus strain used was able to grow by SSF and showed substantial lipase production. Maximum lipase activity attained was 48.6 U/g of dry substrate with a purification fold of 58.09 and a yield of 43.39%. It is concluded from the present study that *A. flavus* could be used as a new potent microbial source of lipase. In further studies, pilot scale production and purification studies will be conducted. Since these microorganisms are generally recognised as safe (GRAS) for food, brewing and pharmaceutical applications, more research is necessary to optimise the fermentative process in order to obtain higher lipase production through this strain.

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