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Full Length Research Paper

Production and characterization of extracellular lipase secreted by *Mucor geophillus*

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Nowadays, the growing interest in microbial lipase production by using the cheapest sources of carbon source, and its potential applications in biotechnological field has shown a remarkable place of prominence among the enzymes, owing to their significant ability to catalyze a wide variety of reactions in aqueous and non aqueous media. The purpose of this study was to enhance the production of lipase by *Mucor geophillus* in submerged culture condition, using molasses mineral medium as a low cost substrate and to increase the economical feasibility attractiveness. *M. geophillus* lipase was also assessed and characterized on the basis of some kinetic parameters. *M. geophillus* secreted higher yield of lipase at approximately 44.56 U.ml^{-1} at 72 h cultivation time. The optimal assay conditions were found at 60 min incubation of 2.0 and 8% enzyme and substrate concentration, respectively. However, lipase was active at 35°C and pH 6.5, but enzyme was found to be stable at about 70°C. From the range of pH 4.0 to 8.0, KCl enhanced the lipase activity, and had higher specific lipase activity towards the olive oil than other substrates.

Key words: Lipase, *Mucor geophillus,* submerged fermentation, characterization.

INTRODUCTION

Lipases or triacylglycerol acylhydrolases (E.C. 3.1.1.3) are enzymes belonging to the hydrolase group that has remarkable catalyzing activity of insoluble triacylglycerols to generate mono, diacylglycerols, glycerol and free fatty acids. Except for their natural function, lipases can catalyze esterification, interesterification and transesterification reactions, and are also involved in acidolysis, aminolysis and alcholysis in aqueous and non-aqueous media (Sharma et al., 2001; Houde et al., 2004). Lipases have interesting characteristics, such as action under mild conditions, stability in organic solvents, high substrate specificity and regio-and enantio-selectivity (Snellman et al., 2002). The great potential for industrial applications of lipases comprises the industry of additives for flavor modification, fine chemistry for ester synthesis, detergents for fat hydrolysis, wastewater treatment for decomposition and removal of oleaginous substances, leather for removal of fat from animal skin, as well as the pharmaceutical and medical area (that is, medicines, digestive aids and enzymes for diagnosis) (Burkert, 2002; Burkert et al., 2004). The inverse processes of glycerol synthesis and transesterification have also great importance, especially in food and cosmetics industry (Paiva et al., 2000). Fungi are one of the most important sources of lipases for industrial applications and nowadays lipases are being produced extracellularly via submerged or solid-state fermentations by several fungal species (De-Azeredo et al., 2007). Recently, microbial lipases assumed a prominent place in the world enzyme market, evidenced by an increase in the amount of information reported in literature, which contemplates an average of 1000 publications per year. After proteases and amylases, lipases are considered the third greatest group in the sales market, moving billions of dollars and showing their versatility of application, which makes them especially attractive for industrial applications (Snellman et al., 2002; Hasan et al., 2006). The use of agro-industrial residues or by-product as substrate for lipase production by submerged fermentation can significantly reduce the final price of the enzyme, and also add value to materials of low cost in the market (Casthilo et al., 2000).

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On the basis of these aspects, the purpose of this study was to evaluate the production of high value lipase by *Mucor geophillus* using agro-industrial by-product (molasses) as a cheap substrate in submerged culture condition. The partial characterization of crude enzymatic extract from fermented molasses mineral medium can facilitate the reduction of industrial scale production cost.

MATERIALS AND METHODS

The *M. geophillus* was isolated from the soil of the University of Sindh, Jamshoro and was grown in the Enzyme and Fermentation Research Laboratory, Institute of Biotechnology and Genetic Engineering. The stock culture was maintained on Czepaks agar, while the sterilized slants were inoculated with *M. geophillus*. After inoculation, the slants were incubated at 27°C to obtain luxuriant growth. The isolation and maintenance of pure culture of *M. geophillus* was done from soil sampling as described by Alexander (1977). *M. geophillus*, a genus of thermophillic mould fungi commonly seen on dead and decaying organic matter, was proposed by Oudemans and Koning (1902). This genus belongs to kingdom thallophyta and is included in order *Mucorales* and family *Mucoraceae* (Abbott, 1923). Mycelium is snow white and is very tardily gray, with pale olive. Sporangiophres is simple or branched in cymes, carrying two to three branches of sporangiablobes, at first yellow, and then olivaceaus, leaving a collar after the destruction of the membrane. It is 50 to 350 µ in diameter, and is found with small wall warts. Columella globose is a voluminous, pale gray chalmydospores found on the branches of the mycelium and is 20 µ in diameter, at a time when there is more or less extended series. However, zygospore is like chalmydospores and is about 30 µ in diameter (Abbott, 1926).

Cultivation condition

Sugar cane molasses were diluted in a 5% concentration (50 g total sugar per 100 ml molasses) with sterile double distilled water and incorporated into the fermentation mineral medium (www.pakistanmolasses.com), after which 50 ml of 5% molasses containing the fermentation medium was taken in 250 ml conical flask and the initial pH of the medium was maintained at 6.0. Flasks were plugged with cotton wool and were then autoclaved at 1.5 kg /cm^2 for 20 min, before they were cooled at room temperature. The surface of seven days stock culture of *Mucor geophillus* was gently rubbed with sterilized wire loop and 10 ml of sterile distilled water, containing 0.01% (v/v), after which Tween-80 was added (Schwermann et al., 1994). The cooled sterilized media was inoculated with spore suspension (1×10^8 per ml) of *M. geophillus*. The inoculated flasks were incubated in an orbital cooled shaking incubator (Gallenkamp) at 30±2°C. The culture broth was separated from mycelium after an interval of 24 h incubation period through Whatman No 1 filter paper. The quantity of mycelium was estimated after washing with distilled water and was dried at 105 to 110°C in a hot oven until a constant weight was obtained.

The following ingredients were used for the preparation of the culture medium as reported by Burrel et al. (1966). The fermentation medium was composed of (g/L) KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $CuSO_4.5H_2O$, 0.0023; $FeSO_4.7H_2O$, 0.0063; $ZnSO_4.7H_2O$, 0.0011; $MnCl_2.4H_2O$, 0.0035; $CaCl_2.2H_2O$, 0.0467; and NH_4NO_3, 2.4 that were used for the production of lipase.

Lipase assay

Lipase activity was measured by the olive oil method (Freire et al., 1997). The reaction mixture containing 2.5 ml of substrate (10% olive oil emulsion with 2% gum Arabic in universal buffer pH 6.5) and 2.5 ml enzyme solution was incubated at 37°C for 1 h in an incubator that was shaken at 200 rev./min. The reaction was stopped by the addition of 5 ml of methanol: chloroform (2: 1 v/v). A unit of lipase activity was defined as the amount of enzyme required to release 1 µ mole of free fatty acids per hour under the assay conditions.

Determination of reducing sugars and total protein

The concentration of reducing sugar from the culture broth was determined by dinitrosalicylic acid (DNS) method (Miller, 1959) at 450 nm, while the total protein contents were estimated from the fermented medium by the method of Lowery et al. (1951) at 750 nm.

Substrate specificity

Substrate specificity towards 10% of rape seed oil, olive oil, caster oil, tripalmitin, tristearin, and tributyrate was investigated.

Effect of substrate and enzyme concentration

The effect of substrate concentration was observed for the rate of enzymatic reaction of lipase by using olive oil as a substrate of various concentrations ranging from 2 to 14%, whilst the effect of enzyme concentration (0.25 to 2.0 ml culture broth) on the rate of enzyme reaction was studied with 8% olive oil as a substrate and incubated at 35°C for 1 h.

Effect of pH and temperature on enzyme activity

The pH effect on lipase activity was identified by measuring enzyme activity at various pH(s) using universal buffer between the ranges of 3 to 10, by means of olive oil as a substrate. The temperature effect on activity was identified by measuring the relative activity at specified temperature in the range of 25 to 45°C.

Effect of pH and temperature on enzyme stability

The pH effect on enzyme stability was evaluated by measuring the residual activity at 37°C for 15 min at various pHs ranging from 3 to 10 using universal buffer. For the determination of the effect of thermal activity, residual activity was measured after incubation of enzyme at various temperature ranging between 30 and 80°C for 5 to 30 min.

Effect of inhibitors or activators on lipase activity

The effect of metal, salts and chemical reagents on lipase activity was investigated by using 5 mM of KCl, NaCl, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $AgNO_3$, $Hg(NO_3)_2$, mercaptoethanol, cystine, sodium deoxycholate (SDC), sodium dodecylsulphate (SDS), Tween–80, Triton–X–100, O–phenonthroline and ethylenediaminetetraacetic acid (EDTA).

RESULTS AND DISCUSSION

The aim of this study was to utilize agro industrial by-product (sugar cane molasses) as a carbon source to

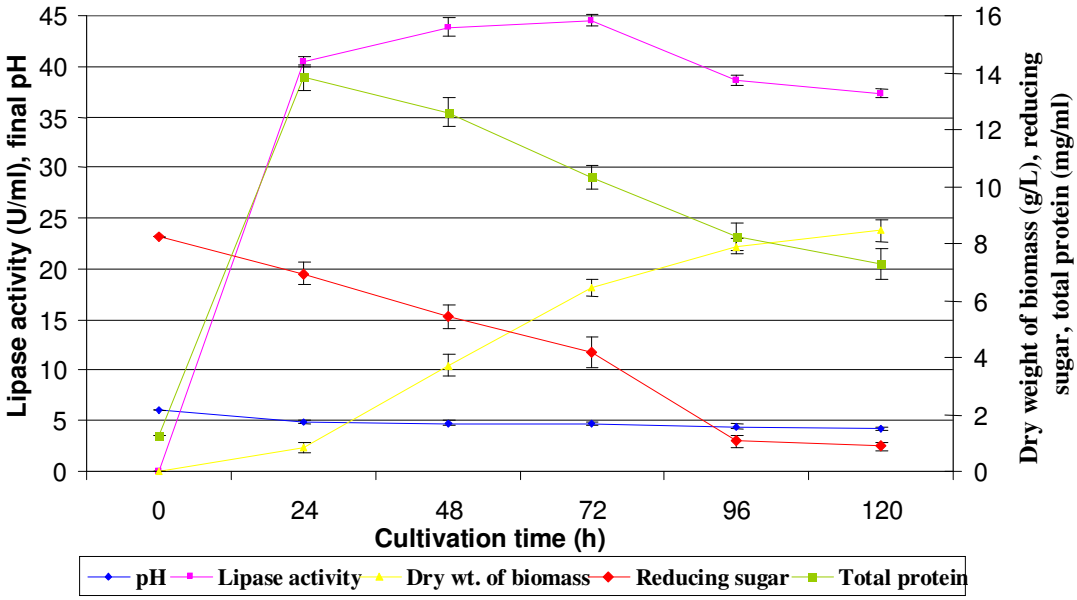

Figure 1. Lipase production by *M. geophillus* grown on 5% molasses at 30 ± 2°C

enhance the production of lipase by *M. geophillus* in submerged culture condition at optimized conditions and characterized it on the basis of enzyme kinetic parameters. There is also very little research reports on the production and characterization of lipase by M. geophillus.

M. geophillus secreted the higher yield of lipase for approximately 44.56 Units/ml at 72 h when it was grown on 5% molasses mineral medium at different cultivation times at 30±2°C. It was observed that the utilization of reducing sugar and secretion of total protein contents increased with the passage of time during the submerged culture condition. The pH value of fermented medium was found towards the acidic side, whereas mycelial biomass was rapidly stimulated throughout the fermentation period (Figure 1). Pinheriro et al. (2008) studied lipase production from *Penicillium verrucosum* and obtained a maximal enzyme yield of 3.22 $U.mL^{-1}$ at 96 h and 2.63 $U.mL^{-1}$ at 72 h in conventional and industrial fermentation medium, respectively. Mahadik et al. (2004) observed less amounts of lipase yield (4.0 and 13 $U.mL^{-1}$) by *Aspergillus niger* and *Penicillium restrictum* in submerged fermentation condition. *Candida rugosa* also secreted low amount of lipase (1.84 $U.mL^{-1}$) after 66 h and 12.55 $U.mL^{-1}$ after 72 h; this was reported by Puthli et al. (2006) and Benjamin and Pandey (1995) separately. Furthermore, *Mucor griseocyanus* produced 0.113 $IU.ml^{-1}$ in optimized fermentation condition (Armas et al., 2008), whilst in this study, *M. geophillus* secreted 44.56 $U.mL^{-1}$ lipase activity at 72 h, which is higher in comparison to the reported work, in which lipase production was evaluated by different fungal species.

M. geophillus secreted lipase was characterized in terms of the effect of time on the activity of lipase at various time periods. The rate of enzymatic reaction increased with the increase of time and reached a maximum activity at 60 min incubation period before it declined (Figure 2). The declination after optimal time period may be suggested due to the presence of other enzymes in the culture broth or deactivation due to prolong incubation (Murray et al., 2003).

The hydrolytic activity towards various natural oils (olive oil, rape seed oil and castor oil) and triacylglycerols (tributyrin, tristearin and tripalmitin) was checked and the results are shown in Figure 3. *M. geophillus* lipase enzyme had an activity towards not only triacylglycerols, but also natural oils, while 100% higher hydrolytic activity was observed towards the olive oil. The main component of olive oil is 70% oleic acid and it is suggested that this specificity is consistent with pure triacylglycerols containing oleic acid than other natural oils which possess different fatty acids in their chain length. Thus, the enzyme was found to have the ability to catalyze all triacylglycerols and olive oil (oleic acid), which possess chain length of four to 18 carbon atoms. The hydrolytic pattern of the enzyme may be affected by the degree of unsaturation of fatty acids. Similar results were seen in the study of Supakdamrongkul et al. (2010) who observed that *Nomuraea rileyi* lipase which hydrolyzed all natural oils as well as all synthetic triacylglycerides, were used in the experiment. It has also been reported earlier by some other researchers who described the capability of lipases that such enzymes catalyze acylglycerols with acyl chains of more than 10 carbon atoms (Jaeger et al., 1999). Furthermore, surface pressure and lipid distribution of such enzymes may affect substrate specificity and activity (Jaeger et al., 1994) by substrate-binding interactions (Hiol et al., (1999).

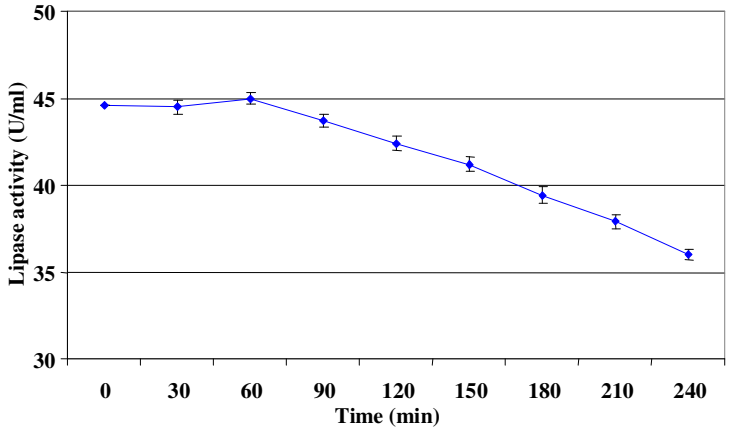

Figure 2. Effect of time on lipase activity produced by *M. geophillus*. The enzyme was measured at pH 6.5, and 37°C.

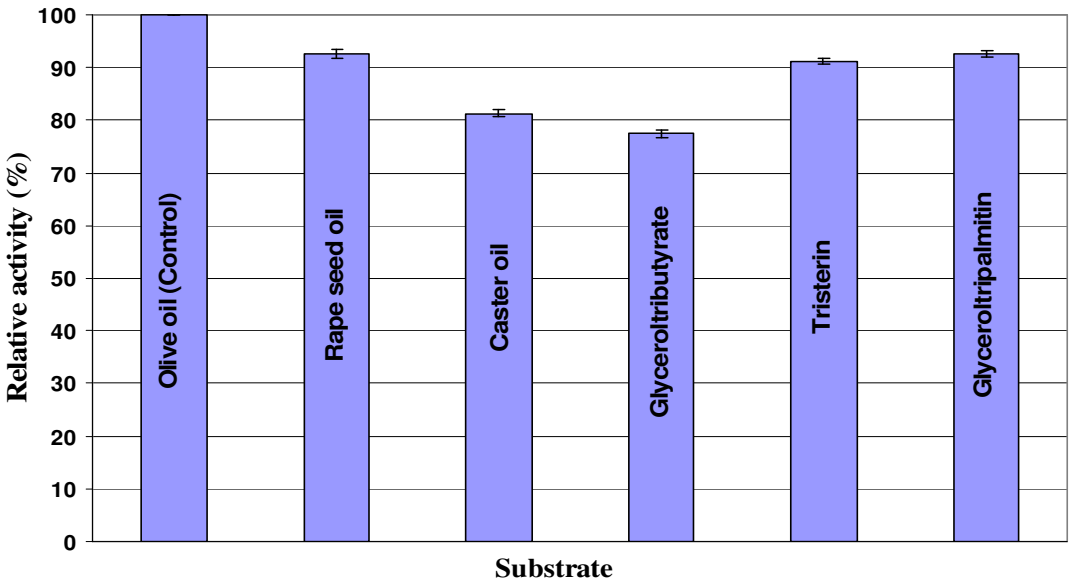

Figure 3. Effect of substrate specificity on lipase activity produced by *M. geophillus*

The influence of substrate concentration on the rate of hydrolytic activity of lipase was checked with various substrate (olive oil) concentrations ranging from 2 to 14%. The enzyme had high activity at 8% substrate concentration, but it retained lipase activity with the increase of substrate concentration as illustrated in Figure 4. The declination, after optimum concentration may be due to change in enzyme and substrate ratio (Murray et al., 2003). Various lipase enzyme (culture broth) concentrations (0.25 to 2.0 ml) of *M. geophillus* were used for the effect of hydrolytic activity. The rate of lipase activity was stimulated with the increase of enzyme concentration up to 2.0% ml as shown in Figure 4. It is reported that if the substrate is tremendously excess and other conditions are fixed, the rate of an enzymatic reaction is proportional to the enzyme concentration (Murray et al., 2003).

The effect of pH on the lipase activity of *M. geophillus* was investigated. In this study, the pH range (3.0 to 10.0) of the substrate was maintained with the universal buffer. The enzyme was most active at pH 6.5, but retained over 47.28% of its activity at pH 10. When it was above pH 9.0 and below pH 6.0, the activity dropped sharply. Lipase activity was found to be 82 and 60% at pH 6.0 and 9.0, respectively. On the other hand, the effect of pH stability of lipase was checked at 35°C by using the same buffer with the same range. The enzyme mixed with different pH values was incubated for 10 min and after mixing the substrate, the enzyme assay was carried out by the standard method as described earlier. The lipase activity

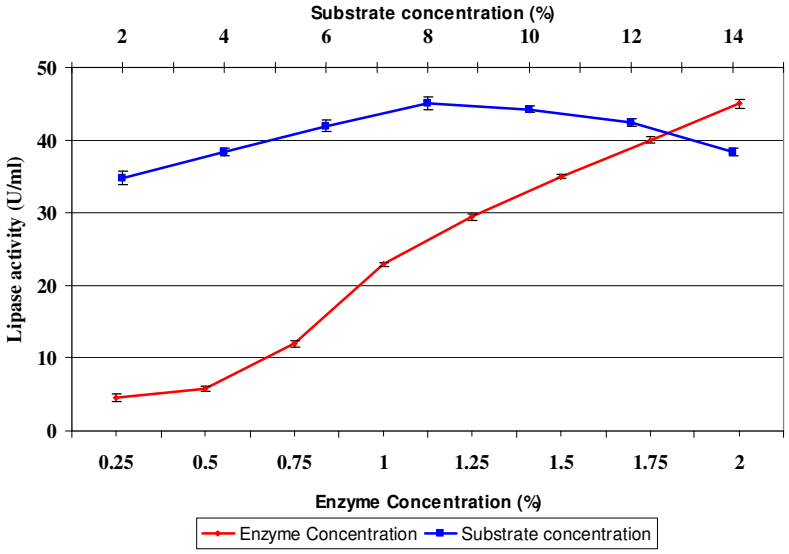

Figure 4. Effect of substrate (olive oil) and enzyme concentrations on lipase activity produced by *M. geophillus.* The enzyme was measured at pH 6.5, and 35°C.

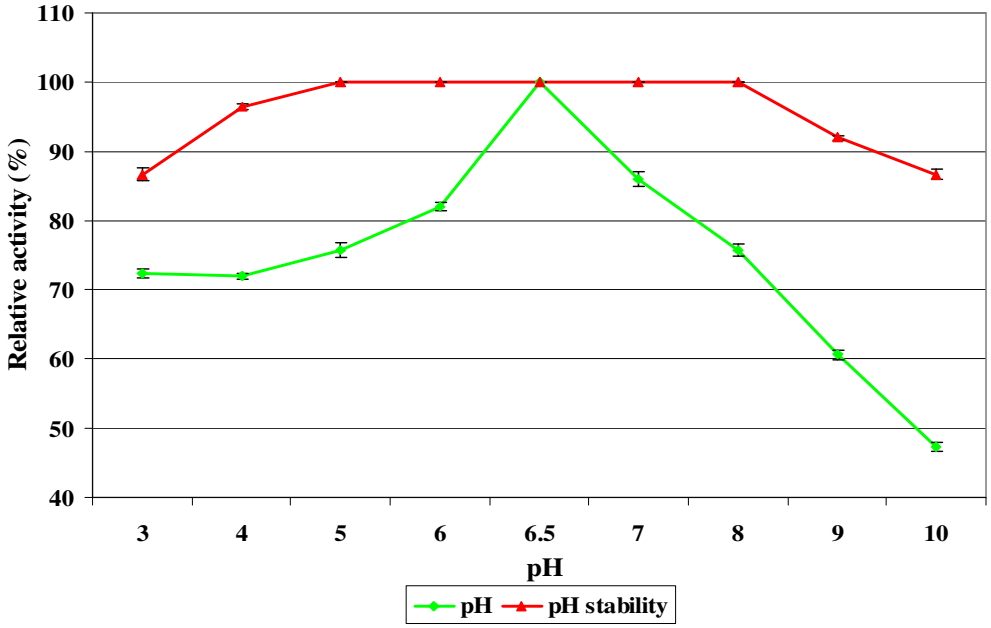

Figure 5. Effect of pH and pH stability on lipase activity produced by *M. geophillus.*

remained with more than 86% activity between pH 3.0 and 4.0, while an activity that was less than 86% was retained at pH 10. The lipase activity was found to be stable between pH 5.0 and 8.0 as represented by the data in Figure 5. Gutarra et al. (2009) reported that crude lipase showed high activities at pH 4.0 and 5.0 from *Penicillium sp*, while Brozzoli et al. (2009) reported that the pH stability of most fungal lipases were found in the range of 6.0 to 8.0 and were unstable above pH 8.0.

The effect of temperature on the rate of lipase reaction was examined at various temperatures ranging from 25 to 45°C as illustrated in Figure 6. The enzyme activity increased with an increase in temperature and reached optimum at 35°C and then fell with the increase of further temperature. An increase in temperature increased the number of effective collision between the enzyme and

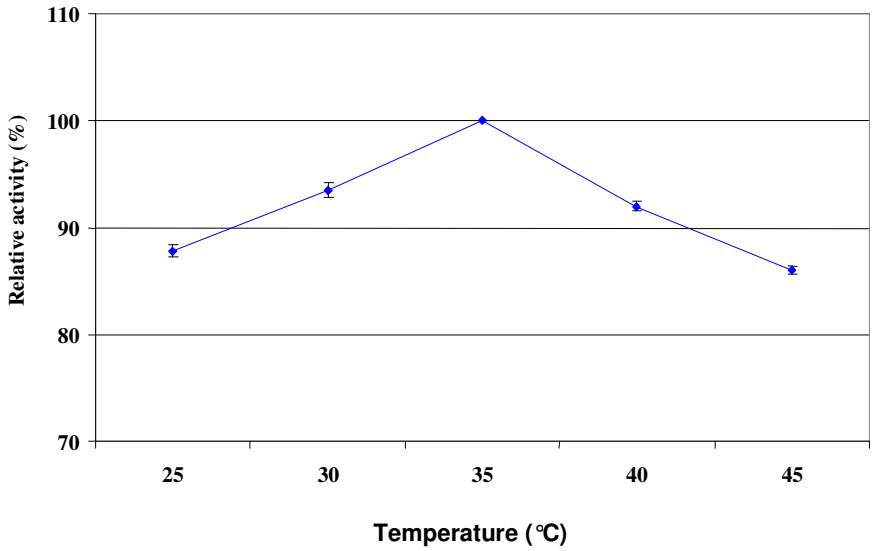

Figure 6. Effect of temperature on lipase activity produced by *M. geophillus*. The enzyme was measured at pH 6.5.

substrate to form the activated complex and thus the rate of reaction increased. There is a limit to the increase in enzyme activity with the increase in temperature. When the rate of enzyme catalyzed reactions is measured at several temperatures, there is an optimal temperature at which the reaction is most rapid, but when it is above that temperature, the reaction rate decreases sharply mainly due to the denaturation of enzyme by heat (Murray et al., 2003). When the effect of temperature and pH were compared with some other fungal species secreted lipase in the submerged fermentation condition reported in the literature, it was observed that the maximum activity was exhibited at 37°C and pH 5.0 (Sztajer et al., 1992), whilst it was also reported that most of the *Penicillium* species secreted lipase showed optimum activities between the range of 25 and 45°C, except *Penicillium aurantiogriseum* lipase optimum activity at 60°C and pH 8.0 (Costa et al., 1999; Tan et al., 2004; Lima et al., 2004).

The assessment of thermal stability of lipase from the culture broth of *M. geophillus* was checked by measuring the residual activity after heating the enzyme at various times (from 5 to 30 min) and at various temperatures (from 30 to 80°C). After heating, the enzyme sample was chilled and the remaining activities were observed by the standard assay method. It was observed that the enzyme was stable up to 70°C and it retained more than 50 and 70% activity at 60°C. When the lipase was heated at 80°C at various times, the activity (about 95%) was lost within 5 min as shown in Figure 7. According to these results, it is noted that lipase is a thermo stable enzyme. Gutarra et al. (2009) stated that high activity of crude lipase from *Penicillium sp*. was recorded and was stable at 35 to 60°C. It is stated by several workers that most mesophilic fungi show low thermal stability above 40°C (Sztajer et al., 1992) while this study reported that the crude lipase was secreted by *M. geophillus* in submerged culture condition; it exhibited good thermal stability as well than some other thermo tolerant fungal and bacterial species such as *P. aurantiogriseum* lipase having optimum activity at 60°C, but showed low heat stability at a temperature above 28°C and retained 32% activity after 30 min at 50°C (Lima et al., 2004). *Penicillium wortrnii* secreted the lipase that was reported to be a moderate thermostable enzyme and retained 55% activity at 50°C after 1 h incubation (Tan et al., 2004). Thermostability of crude lipase produced by helophillic *Archea natronococcus* showed more than 90% activity at 50°C after 1 h (Boutaiba et al., 2006). Similarly, high thermostability of the lipase produced by several bacterial species reported in the literature, such as *P. aurenginosa*, retained 30 and 75% activity after 15 min at 60 and 70°C incubation (Karadzic et al., 2006).

The effect of various chemical reagents or metal ions in the presence of universal buffer pH 6.5 on lipase activity was investigated when incubated at 35°C for 10 min prior to the assay of the remaining activity. The final concentration of each chemical or metal ion in the reaction mixture was 5 mM as shown in Figure 8. It was observed from the experimental data that KCl stimulates the lipase activity in comparison to others. SDC, $CaCl_2$ and $AgNO_3$ slightly inhibited the activity, whereas cysteine, mercaptoethanol, SDS and $ZnCl_2$ strongly inhibited the lipase activity. In contrast, Mohamed et al. (2011) reported that *Mucor racemosus* lipase showed no significant effect on enzyme activity with Ca^{2+}, $CO^{2+,}$ K^{+} and Hg^{2+}.

Metal ions perform essential catalytic and structural roles in enzymes. Over one fourth of all known enzymes contain tightly bound metal ions that are required for enzyme activity. Many enzymes lost their activity through normal purification procedures due to the loss of metal

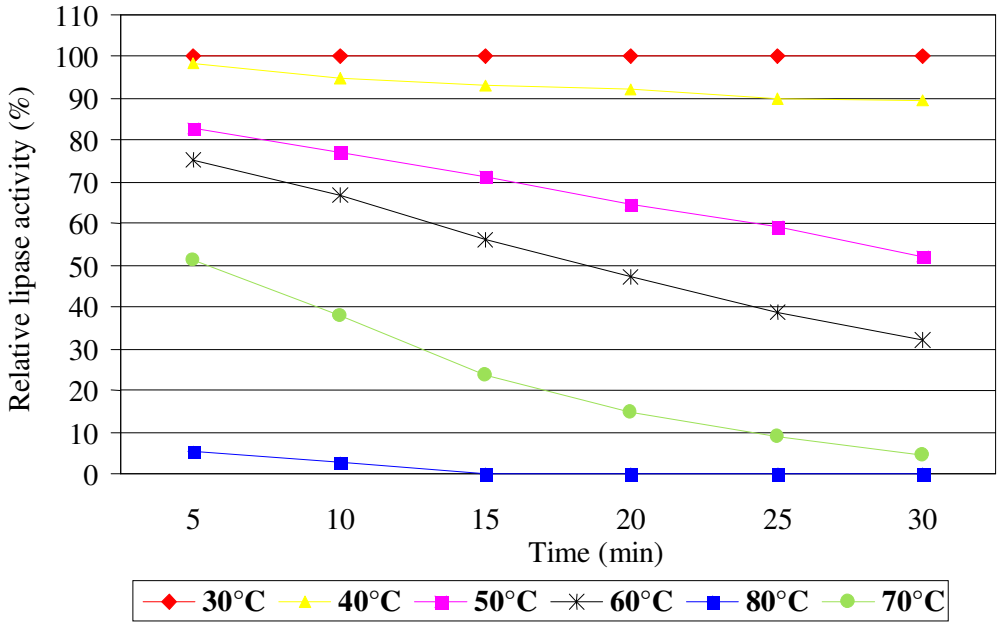

Figure 7. Effect of thermal stability at various time periods on lipase activity produced by *M.* geophillus.

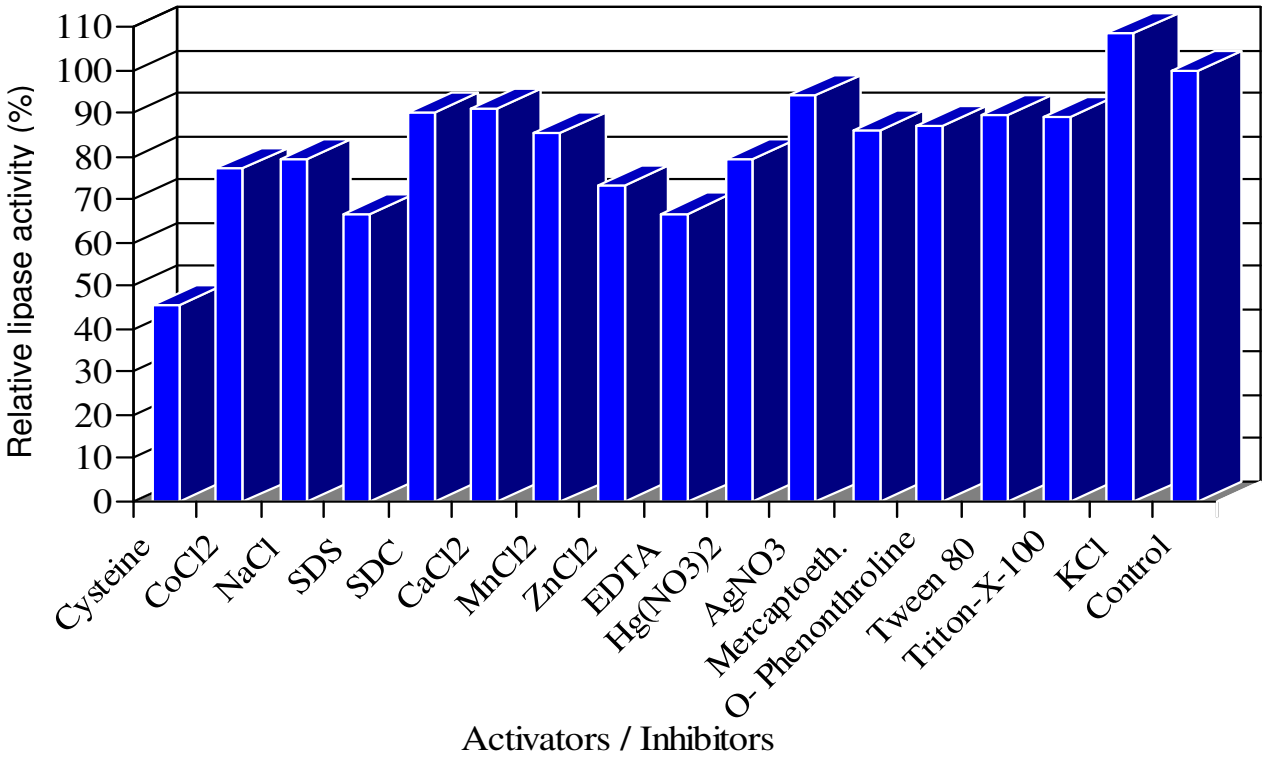

Figure 8. Effect of various activators and inhibitors on lipase activity produced by *M. geophillus*.

when purified in the presence of chelating agents. Enzyme activity can be restored only after addition of metal ions (Murray et al., 2003). However, enzymes were inhibited by divalent ions of heavy metals due to direct attack on the catalytic side, which either block the active side or cleavage the bonds formed in-between the active

amino acids. Thus, this type of inhibition clearly indicated that the amino acid is present at the active site. Lee and Rhee (1993) also reported two mechanism systems of ion action on enzyme activity. The first one is direct action of the ion at the catalytic site on many other enzymes, while the second one is the specific action, resulting from the formation of complex ions between ionized fatty acids and metal ions that may change their solubility and behavior at interface. However, strong inhibition was observed by Supakdamrongkul et al. (2010) in their experiment of *Nomuraea rileyi* lipase with some divalent metal ions like CO^{2+} and Mn^{2+}. In our experiments, all surfactants also inhibited the *M. geophillus* lipase activity, and it was observed that the results were similar to those of Hiol et al. (1999, 2000) and Yu et al. (2007), who reported that Triton X-100, SDS and other surfactants inhibited the *M. hiemalis, R. oryzae* and *Y. lipotica* lipases activities.

Conclusion

The highest production of lipase (approximately 44.56 Units / ml) was recorded by *M. geophillus*, when it was grown on 5% molasses at 72 h. According to the kinetic study of *M. geophillus* secreted lipase, it was observed that the rate of catalytic activity was found at 60 min incubation. The rate of lipase activity was increased with the increase of enzyme and substrate concentration up to 2.0 and 8%, respectively, while the higher specific lipase activity was observed towards the olive oil than other substrates. Its optimum temperature was found at 35°C, but the enzyme was found to be stable up to 70°C. It is concluded that this enzyme is thermo stable, whereas its optimum pH was noted as 6.5 and its pH stability was observed in the range of pH 4.0 to 8.0. KCl stimulated the lipase activity in comparison to other metal ions and reagents. It is concluded that this enzyme has several differences regarding its production and properties in comparison to a previous reported lipase production by filamentous fungal species in submerged culture condition, showing greater thermal stability, thereby enhancing specific activity and high optimum conditions. Thus, the results of this study show that the lipase of *M. geophillus* has a very attractive biotechnological potential on the basis of its wide specificity at elevated temperatures, while the other characterized properties and high yield of lipase at low production cost can infer to its use for different purposes.

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