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Characterization of Lipase from Wild (LPF-5) and Mutant (HN1) Strain of *Aspergillus niger*

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A B S T R A C T

Lipase is one of the most imperative industrial enzymes and has a variety of applications in various industries. In the present study, lipases obtained by submerged cultivation of wild (LPF-5) and mutant (HN1) strain of *A. niger* were used and compared for characterization study. Activity and stability of partially purified lipase was determined under different pH, temperature, organic solvents and metal ions. The lipase showed highest activity and stability at pH 7.0 and temperature 35 °C for LPF-5 and 30 °C for HN1 strain. The lipases retained high activity over ranges of temperature (25-50 ºC) and pH (4.0-7.5). Lipase was enhanced by methanol and acetone, while slightly inhibited by butanol (10% v/v). Ca^{2+} appeared to be the excellent inducer of lipase activity. Lipase also showed stability in presence of the other metal ions (Na^{+}, Ba^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} and Mn^{2+}). The lipase of wild and mutant strain retained 10.58% and 14.60% of its activity when pre-incubated with Hg^{2+}, indicating the inhibitory effect of Hg^{2+} on catalytic activity of lipase.

Introduction

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) belonging to the class Hydrolases catalyze hydrolysis of insoluble triacylglycerols (TAGs) to generate free fatty acids, mono and diacylglycerols and glycerol (Das *et al.*, 2016). Lipases are found in both prokaryotes (bacteria and archaea) as well as in eukaryotes (animals, plants and fungi) (Cai-hong *et al.*, 2008). The most broadly applied lipases are microbial due to their stability and chemical properties (Hasan *et al.*, 2009). Although several microorganisms are recognized as potent producers of lipases (extracellular) including fungi, yeast and bacteria but fungal lipases are being used for diverse applications in biotechnological industries (Sharma and Kanwar, 2014). Extracellular secretion of lipases has been well documented for a variety of fungi (yeasts, zygomycetes and hyphomycetes). Filamentous fungi are preferred sources of lipases among the rest of the lipase producing microorganisms. The main producers of commercial lipases mainly are *Rhizopus* sp., *Pencillium* sp., *Aspergillus* sp., *Mucor* sp., *Fusarium* sp., *Candida*

rugosa, *Acremonium alcalophilum* and *Geotrichum candidum* (Jayaprakash and Ebenezer, 2012).

Soil is one of the excellent sources for the isolation of novel lipase producing microorganisms as it gives nutrient rich environment to enable the high proliferation of microorganisms (Shukla and Desai, 2016). About 3000 enzymes are known today but only a certain enzymes are being used in various industries. These are mainly hydrolytic enzymes (secreted extracellularly), which are capable of degrading natural polymers such as cellulose, lipid, starch, proteins and pectin into simpler monomers (Sharma *et al.*, 2015). Following carbohydrases and proteases, lipases are believed to be the third major group based on total sales volume (Ray, 2012). Lipases (and esterases) are capable of catalyzing three types of reactions. The biological action of lipases is reversible. They are capable of catalyzing hydrolysis in an aqueous environment and esterification in a micro aqueous environment, in which content of water is very low. According to the nature of chemical species which react with the ester, transesterification is classified into four subclasses: alcoholysis, acidolysis, interesterification and aminolysis (Sharma and Kanwar, 2014). Lipases found promising application in various industries such as detergent, agrochemical, paper, chemical processing, dairy, pharmaceuticals, oleochemical, cosmetics, polymer synthesis, synthesis of surfactants and personal care products (Ray, 2012).

Lipase characterization would determine its possible utilization in various environments and industries. The optimum pH for the fungal lipases was found to be in the range of 4.0 to 8.0 (Hasan *et al.*, 2009). The majority of the fungi have optimum temperature between 25-30 °C for highest activity of lipase except for certain thermophilic fungi which have optimum temperature range of 45-75 °C (Sharma *et al.*, 2011).

In view of the significance of lipase for numerous applications, we made an attempt to determine the stability of lipase (obtained from wild and mutant strain of *A. niger*) under different pH, temperatures, organic solvents and metal ions.

Materials and Methods

Production of lipase from *Aspergillus niger*

Wild (LPF-5) and hyperproducer nitrous acid mutant strain (HN1) of *A. niger* were obtained from Department of Bioscience and Biotechnology, Banasthali University and utilized for lipase production in submerged fermentation. Spore suspensions were prepared from 7 days old slant culture of both the fungus and 1 mL of this was transferred in 250 mL Erlenmeyer flask containing 100 mL of production medium followed by incubation at 28 $^{\circ}$C, 160 rpm for 42 h. The composition of production medium (g L^{-1}) is as follows: bacteriological peptone, 10; olive oil, 1%; $MgSO_4.7H_2O$, 0.6; KH_2PO_4, 1.0; NH_4NO_3, 1.0 and pH was adjusted to 7.0. At the end of incubation, enzyme containing supernatant was recovered after filtration and centrifugation of culture broth.

Lipase assay

Lipase activity was determined by spectrophotometric technique (using *p*-nitrophenyl palmitate as substrate) (Winkler and Stuckmann, 1979). Upon completion of enzymatic reaction, the quantity of released yellow colour compound (*p*-nitrophenol)

was measured at 410 nm against a reagent blank using reference curve of *p*-nitrophenol (in the concentration range of 2-20 µg mL^{-1} of *p*-nitrophenol in 0.05 M Tris HCl buffer, pH-8.0). One unit (U) of lipase activity was defined as micromole (µM) of *p*-nitrophenol liberated by hydrolysis of *p*-nitrophenyl ester by one mL of soluble enzyme per minute at 35 °C under standard assay conditions.

Partial purification of lipase enzyme

Lipase produced by both the wild (*A. niger* LPF-5) and mutant (*A. niger* HN1) strain was partially purified by ammonium sulfate precipitation (Data is not shown here).

Characterization of partially purified lipase of wild (LPF-5) and mutant strain (HN1) of *A. niger*

The partially purified lipase preparation (obtained at 70% $(NH_4)_2SO_4$ saturation) from both the wild and mutant strain was then characterized to study the impact of pH, temperature, organic solvents and metal ions on the stability and activity of the lipase.

Impact of pH on the activity and stability of partially purified lipase

To determine the pH optima, lipase activity was measured at various pH (4, 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0). The pH of the reaction mixture was adjusted using buffers of different pH. These buffer solutions (20 mM) include sodium acetate buffer (4 and 5), sodium phosphate buffer (6 and 7), Tris-HCl buffer (8 and 9) and sodium bicarbonate buffer (10 and 11) (Sarkar and Laha, 2013).

pH stability was determined by pre-incubation of lipase preparation with the buffers of above mentioned pH. The partially purified enzyme and respective buffer solution were mixed (1:1 v/v) together and incubated for 1 h at 30 ºC (Iftikhar *et al.*, 2011). Remaining enzyme activity (residual enzyme activity) was determined by considering the activity of the enzyme without any pre-incubation as 100%. Residual lipase activity was determined by dividing the activity of each sample by the activity found in the control sample and it was expressed in percentage.

$$\text{Residual activity} = \frac{\text{Activity of sample (U mL}^{-1}\text{) x 100}}{\text{Activity of the control (U mL}^{-1}\text{)}}$$

Impact of temperatures on the activity and stability of partially purified lipase

Lipase assay was carried out at various temperatures (25 ºC, 30 ºC, 35 ºC, 40 ºC, 50 ºC and 60 ºC) in order to determine the temperature optima. Temperature stability in above mentioned temperatures was determined by pre-incubating the lipase sample at respective temperature for 1 h and residual enzyme activity was determined by considering the activity of the enzyme without any pre-incubation as 100% (Iftikhar *et al.*, 2011; Sarkar and Laha, 2013).

Impact of organic solvents on the activity of purified lipase

The impact of different organic solvents viz.: methanol, ethanol, acetone, butanol and isopropanol (10% v/v) was investigated on lipase stability by pre-incubation of enzyme fractions and organic solvent (1:1 v/v) at 37 ºC for 1 h. Thereafter, lipase activity was determined. However, the residual enzyme activity was measured by considering the activity of the enzyme without any organic solvents as 100% (Kumar *et al.*, 2012).

Impact of various metal ions on the activity of partially purified lipase

Impact of different metal ions (at a concentration of 1 mM) on activity of lipase was evaluated by pre-incubating the mixture of enzyme and metal ion solution for 1 h and then residual lipase activity was determined by considering the activity of the enzyme without any metal ion as 100%. The metal ion studied included sodium chloride, calcium chloride, barium chloride, magnesium sulfate, copper sulfate, ferrous sulfate, manganese chloride and mercury chloride which contributed the metal ions, Na^{+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and Hg^{2+}, respectively (Ulker *et al.*, 2011).

Results and Discussion

Impact of temperatures on the activity of partially purified lipase

In the temperature optima profile (Fig. 1), different behaviour by wild (*A. niger* LPF-5) and mutant (*A. niger* HN1) strain was observed. Highest lipase activity by wild (83.42 ± 0.95 U mL^{-1} min^{-1}) and mutant strain (99.51 ± 1.01 U mL^{-1} min^{-1}) was observed at 35 and 30 °C, respectively. For wild strain the activity was highest at 35 °C, followed by 40, 30, 25, 50 and 60 °C and for mutant strain the activity was highest at 30 °C, followed by 35, 25, 40, 50 and 60 °C. Activity of lipase by wild strain at 40 °C, 50 °C and 60 °C was found higher than mutant strain, indicating superior thermostability of lipase of wild strain over mutant strain.

In accordance with the current results, an optimum temperature of 30 °C for lipase activity was reported by Hosseinpour *et al.* (2011) for *A. niger*, Prabhakar *et al.* (2012) for *Rhizopus* sp. and Mahmoud *et al.* (2015) for *A. terreus*. An optimum temperature of 37 ºC which is very close to temperature optima of our wild strain (35 ºC) was reported for activity of *A. niger* lipase by several investigators (Pera *et al.*, 2006; García *et al.*, 2014). Only a few fungal lipases show temperature optima above 40 ºC (Razak *et al.*, 1997). However, Niaz *et al.*, (2013) observed highest activity of *A. niger* lipase at 45 ºC.

Impact of temperatures on the stability of partially purified lipase

Lipase from both the wild and mutant strain retained stability within the temperature range of 25-50 ºC. Wild strain lipase retained almost 100% of its activity at 40 ºC, while lipase of mutant strain showed highest stability at 35 ºC. Lipase of mutant culture retained more than 80% of its activity within the temperature range of 25-40 ºC. Similar to temperature optima pattern, lipase of wild strain was more stable at 40, 50 and 60 ºC than mutant strain, indicating its thermostability over mutant strain (Fig. 2). The lipase activity was greatly decreased by increasing the incubation temperature above 50 ºC; lipase of mutant strain retaining only 25.43% of its activity at 60 ºC. It might be due to that at elevated temperature, rearrangement of hydrogen and ionic bonds in solvated layer could lead to the conformational changes at the active site of enzyme, therefore activity of enzyme is lost. Ultimately, the enzyme becomes denatured.

The present results are in agreement with Maliszewska and Mastelerz (1992) who reported that lipase of *Penicillium* sp. was most active at 40 ºC but unstable above 50ºC. Petersen and Daniel (2006) also demonstrated higher extracellular lipase stability at 40 ºC. Adham and Ahmed (2009) noticed that when lipase of *A. niger* was pre-incubate at 60 ºC for 1 h, the enzyme activity decreased significantly. Sundar and Kumaresapillai (2013) observed that lipase

of *A. niger* retained stability up to 40 ºC with maximum stability at 30 ºC.

Impact of pH on the activity of partially purified lipase

Highest lipase activity was obtained for both the wild (102.77 ± 0.84 U mL^{-1} min^{-1}) and mutant strain (123.32 ± 1.08 U mL^{-1} min^{-1}) when the pH of the reaction mixture was 7.0. For wild strain the lipase activity was highest at pH 7.0, followed by 6.5, 6.0, 7.5, 5.5, 5.0, 4.0, 8.0 and 9.0, while for the mutant strain the lipase activity was highest at pH 7.0 followed by 6.5, 7.5, 6.0, 5.5, 5.0, 4.0, 8.0 and 9.0 (Fig. 3). The activity of lipase from both the strains was found to be higher in the acidic pH range (4.0-6.5) than in the alkaline pH range, which indicates the acidic nature of the lipases.

An optimum pH of 7.0 as found in the present study for the activity of *A. niger* lipase was also reported by other investigators (Hosseinpour *et al.,* 2011; Sundar and Kumaresapillai, 2013; García *et al.,* 2014). Other investigators (Falony *et al.,* 2006; Sarkar and Laha, 2013) noticed an optimum pH of 6.0 for *A. niger* lipase.

Impact of pH on the stability of partially purified lipase

The lipase was found stable within the pH range of 4.0-7.5 with maximum stability at pH 6.5 (Fig. 4). Stability of lipase was higher in the acidic pH range than in the alkaline, which shows the acidic behaviour of lipase. At pH 4.0, lipase of both wild and mutant strain retained 37.08% and 50.39% activity, respectively. It indicates that lipase obtained from mutant strain is more acid tolerant than wild strain.

Adham and Ahmed (2009) reported that activity of lipase was reduced up to 50% when pre-incubated at pH 8.0 for 1 h and Dimitris *et al.,* (1996) revealed that lipase of *A. niger* underwent rapid denaturation in alkaline pH and lost 50% of its activity when incubated for 1 h. These findings corroborated with the present results.

Impact of organic solvents on the activity of purified lipase

Of all the tested organic solvents, methanol increased the residual activity up to 110.52% for wild strain and 128.97% for mutant strain when compared to 100% activity of control of respective strains (Fig. 5). Methanol appeared to be the excellent inducer of the activity of lipase, followed by acetone, isopropanol, ethanol and butanol. Partially purified lipase from both the wild and mutant strain of *A. niger* exhibited good tolerance to most of the tested organic solvents but the lipase of the mutant strain was found to be more tolerant towards the organic solvents than the lipase of wild strain. In agreement with the present results, many workers found methanol as an excellent organic solvent for induction of lipolytic activity. Jayaprakash and Ebenezer (2012) reported that lipase of *A. japonicus* was stable in methanol, acetone, ethanol and hexane with the highest and lowest stability in methanol and butanol, respectively after pre-incubation of 1 h at 40 ºC. Similarly, the lipase of *A. niger* was found stable in methanol, acetone and ethanol, the best solvent being the acetone after pre-incubation of 1 h at 37 ºC (Pera *et al.,* 2006).

Impact of metal ions on the activity of partially purified lipase

Metal ions play an important role in enzymology. Thus, the impact of various metal ions on the stability of lipase was carried out in this study. Among all the metal ions, Ca^{2+} increased the residual activity up to 104.70% for wild strain and 111.24% for mutant strain when compared to 100% activity of control (Fig. 6).

Fig.1 Impact of temperatures on the activity of partially purified lipase by wild (LPF-5) and mutant (HN1) strain of *A. niger*

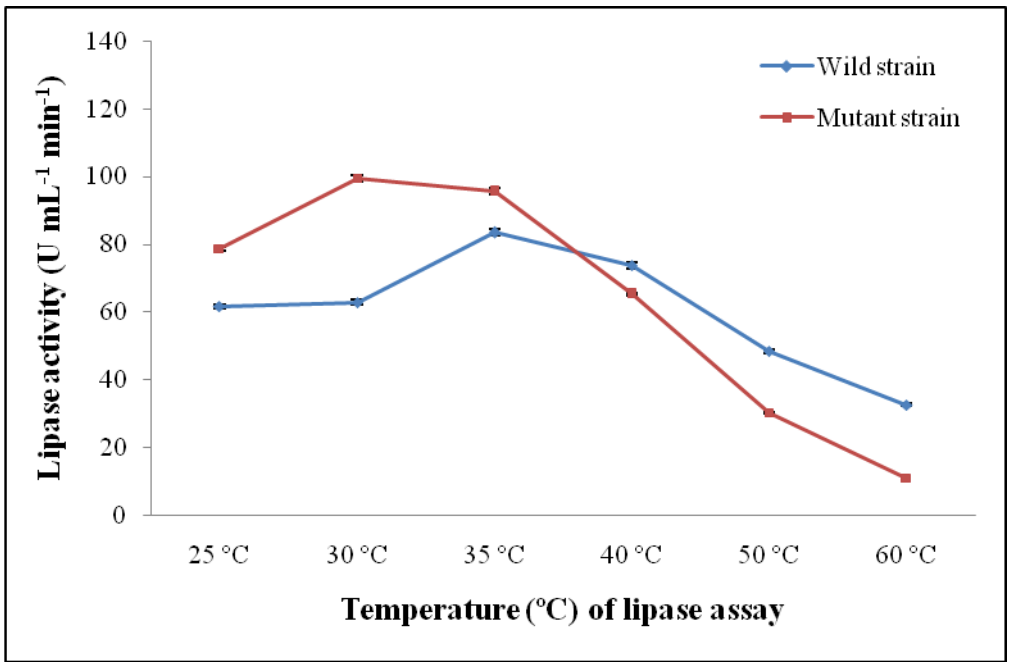


Fig.2 Impact of temperatures on the stability of partially purified lipase by wild (LPF-5) and mutant (HN1) strain of *A. niger*

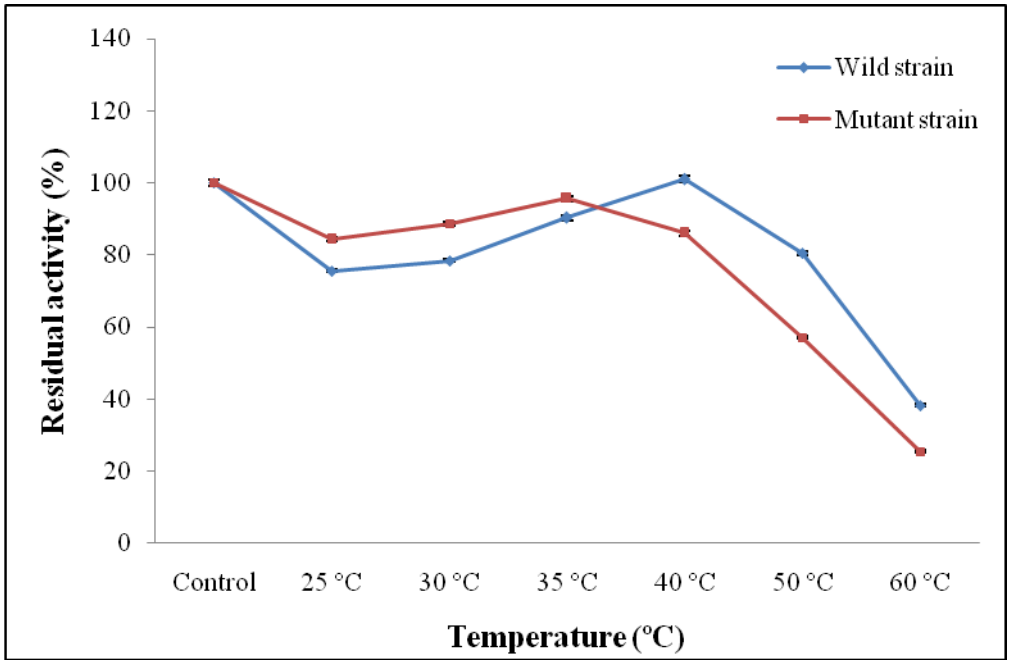


Fig.3 Impact of pH on the activity of partially purified lipase by wild (LPF-5) and mutant (HN1) strain of *A. niger*

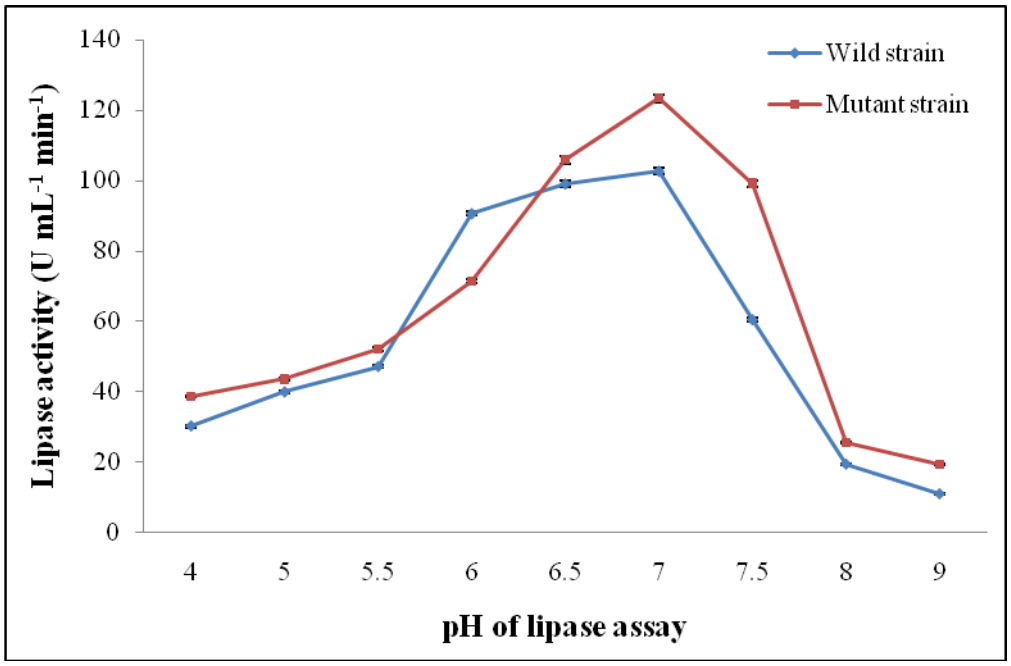


Fig.4 Impact of pH on the stability of partially purified lipase by wild (LPF-5) and mutant (HN1) strain of *A. niger*.

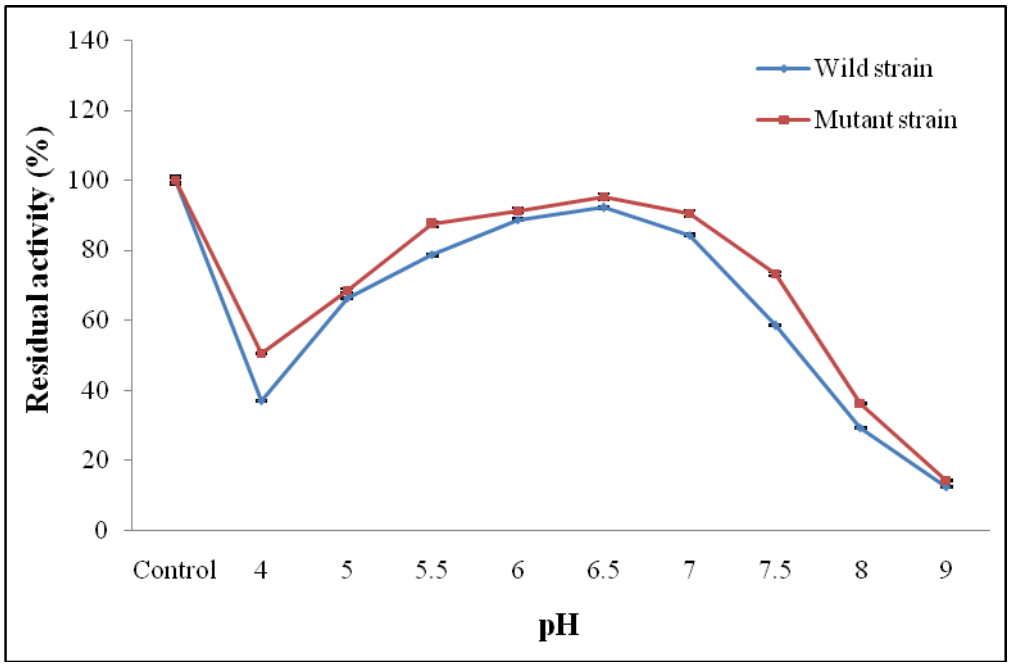


Fig.5 Impact of organic solvents on the stability of partially purified lipase by wild (LPF-5) and mutant (HN1) strain of *A. niger*.

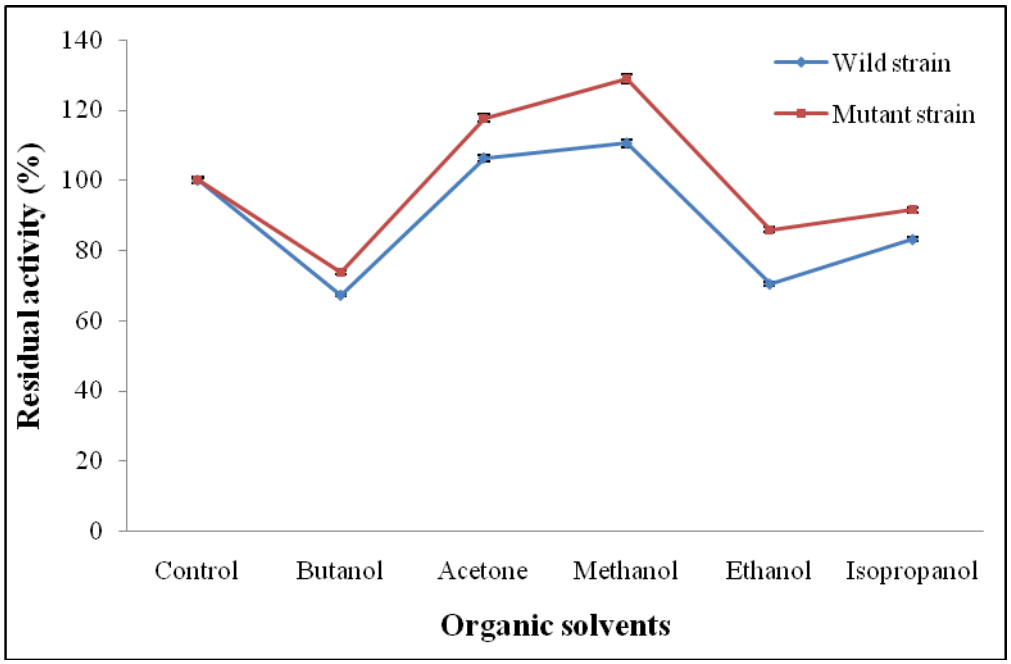
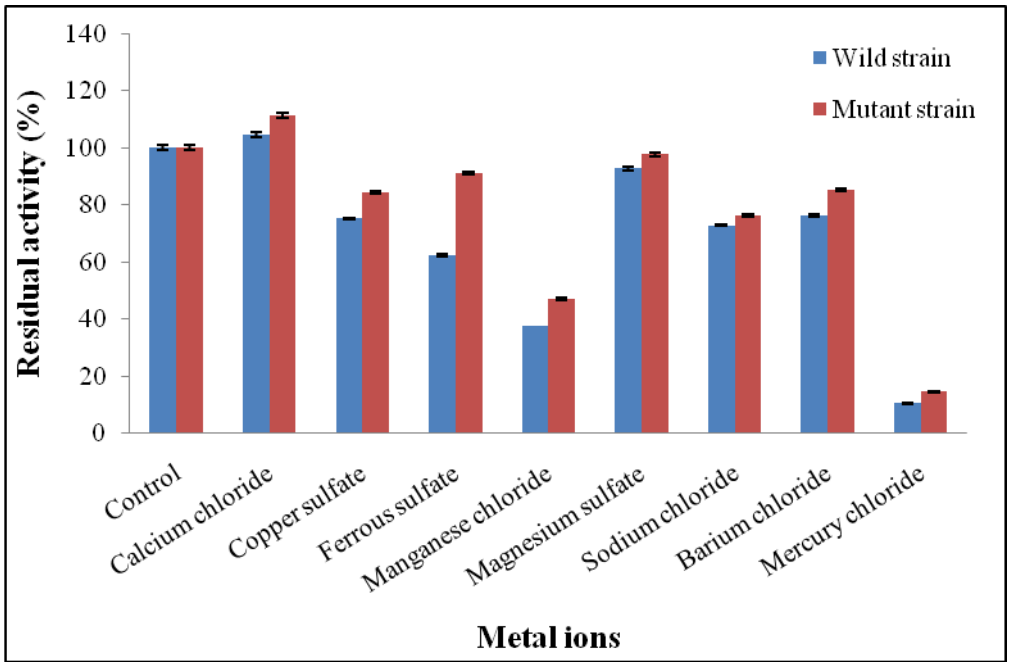


Fig.6 Impact of metal ions on the stability of partially purified lipase by wild (LPF-5) and mutant (HN1) strain of *A. niger*.



The present findings suggest that stimulatory effect of calcium is due to that Ca^{2+} ions bind with enzyme and alter the conformation of enzyme. Now enzyme with modified conformation exhibit greater stability and activity. Ca^{2+} ions appeared to

be the excellent inducer of activity of lipase, followed by Mg^{2+}, Fe^{2+}, Ba^{2+}, Cu^{2+}, Na^{+}, Mn^{2+} and Hg^{2+}. Stability of lipase was found with all metal ions except Hg^{2+}. The residual activity was reached to minimum after pre-incubation with Hg^{2+}.

Adham and Ahmed (2009) reported that Ca^{2+} increases the activity of *A. niger* lipase up to 170%, while Hg^{2+} and K^{+} decreases the residual activity of lipase up to 18% and 22%, respectively as compared to the 100% activity of control, which matches to the present results. Stimulatory effect of Ca^{2+} on lipase activity after 1 h pre-incubation was also reported by Hosseinpour *et al.,* (2011) for *A. niger* and Das *et al.,* (2016) for *A. tamarii* JGIF06.

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