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PARTIAL PURIFICATION AND CHARACTERIZATION OF LIPASE PRODUCED BY *SACCHAROMONOSPORA AZUREA* AP11/18.

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Abstract - An extra cellular lipase produced by *Saccharomonospora azurea* AP11/18 was purified by ammonium sulphate precipitation, ultra filtration, dialysis followed by gel exclusion chromatography on Sephacryl S-200 using Phosphate buffer (pH 8.0). The lipase is made up of two polypeptide chain having molecular weight 50kDa and 42kDa as determined by gel filtration and by SDS- polyacrylamide gel electrophoresis. The Km and Vmax values of lipase were found to be 21.5 μMoles and 17.85 U/mg respectively. The purified lipase was active within the pH range of 6.0-13.0, with an optimum pH of 11.0, and within the temperature range of 40-80°C, with optimum temperature for the hydrolysis of pNPP at 50°C. The hydrolytic activity of the enzyme was enhanced by Mn+2 but strongly inhibited by heavy metals Hg+2 as well as EDTA. While no effect in the presence of Cu+2 and Ca+2 salts.

INTRODUCTION

Lipases which hydrolyze esters of fatty acids, are therefore carboxyester hydrolases and classified as 3.1.1. (Patil *et al.,* 2011). Lipases catalyze both the hydrolysis and the synthesis of long-chain acylglycerols which can usually be precede with high regioselectivity and enantioselectivity (Jaeger *et al*., 1999). Lipases under appropriate reaction conditions, promote ester formation through reaction of acids and alcohols (esterification) or of esters with acids (acidolysis), alcohols (alcoholysis), or other esters (interesterification) (Gunstone, 1999; Gupta *et al*., 2004; Hedfors *et al*., 2010; Zhang *et al*., 2011).

Lipolytic enzymes are currently attracting significant attention because of their biotechnological potential. Lipase is reported to be a thermostable enzyme produced by thermophilic as well as mesophilic organisms. Different kinds of alkalophilic microorganisms, including bacteria belonging to the genera *Bacillus, Micrococcus, Pseudomonas, Streptomyces*, and *Thermoactinomyces*, and eukaryotes, such as yeasts and filamentous fungi, have been isolated from a variety of environments. Thermophilic Monosporic Actinomycetes with single spores on aerial hyphae are included in the genera *Thermoactinomyces, Thermomonospora* and *Saccharomonospora*. While Thermoactinomyces has the presence of true bacterial endospores, *Saccharomonospora* shows the presence of arabinose and galactose in cell hydrolysates (McCarthy and Cross, 1984). *Saccharomonospora azurea* AP 11/18 is monosporic actinomycetes.

Different strategies are being used for the purification of various fungal lipases (Saxena et al., 2003). The extracellular enzymes are the most thoroughly characterized and are produced in much higher amounts (Haas & Joerger, 1995). The optimum pH and temperature for activity of the enzyme were 7.0 and 40°C, respectively (Iftikhar and Hussain, 2002). The lipase was stable in the pH range of 4-9 and at 45°C for 15 min as reported by Hiol *et al*., (2000). Thermostable lipases from various sources have been purified and characterized (Adams and Kelly, 1998; Handelsman and Shoham, 1994; Kim *et al*., 1998; Sugihara *et al*., 1992).

In the present study, *Saccharomonospora azurea* AP 11/18, isolated from a oil industry unit of Kadi

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region (Dist. Mehsana) situated at the North Gujarat, western coast of India, was chosen as the starting material for purification and characterization of an extracellular lipase. We report methods for lipase purification from *Saccharomonospora azurea* AP 11/18 culture broth, and characterization with respect to its biochemical properties intended for use in biotechnological application.

MATERIALS AND METHODS

Microorganisms and culture conditions

A lipase producing strain of *Saccharomonospora azurea* AP 11/18 was isolated on modified Bennet's agar incubated at 40ºC for 96 hrs.

50 mL of sterilized Bennet's broth in 250mL Erlenmeyer flasks was inoculated with 6 mm disc culture and incubated at orbital shaker at 140 rpm and 40°C for 96 hrs. When the optimum growth (O.D 2.0 at 600 nm) was achieved, 1.0% of this growth was used for inoculating 50 mL lipase production medium (1.65g %, Caster oil, 1.10g% Meat extract and 0.06g% Ammonium sulphate) in 250 mL flasks keeping growth conditions the same as in growth phase. After 96 hrs. of lipase production, broth was collected. The extraction of lipase was carried out by filtration of broth using whatman filter paper no.1.

Spectrophotometric assay of lipases

Extracellular lipase activity was assayed spectrophotometrically using p-nitro phenyl palmitate (p-NPP) as substrate according to the method reported by Savitha *et al*., (2007). One lipase unit (U) is equal to the amount of enzyme that liberated 1 µ mole p-nitro phenol per 60 min. On the bases of lipase production in submerged culture, 2 best isolates were selected for further studies. Calculations of lipases units was done by the using the standard curve of p-nitro phenol.

Purification of *Saccharomonospora azurea* AP11/18 lipase

Purification of lipases was carried out by ammonium sulfate precipitation, dialysis and gel filtration chromatography (Saxena *et al*., 2003).

Ammonium sulphate precipitation

Crude enzyme was subjected to ammonium sulfate precipitation by the method of Saxena *et al*., (2003). The enzyme filtrate was adjusted to 85% Ammonium sulfate saturation. It was subjected to ultra filtration.

Dialysis of enzyme

After ultra filtration, precipitates of lipase were dissolved in 0.2M phosphate buffer pH.8.0. Dialysis was carried out using dialysis membrane-50 (Hi media) in 0.2M phosphate buffer pH.8.0 for 24 hrs. Dialysed and removed factions of buffers were checked for presence of lipase. After dialysis, sample was further purified with Gel filtration.

Gel filtration chromatography

A column of Sephacryl S-200 (Pharmacia) was prepared by the method of Samsumaharto, (2008). The flow rate was adjusted to 0.5 mL/min. and fractions of 2 mL were collected and assayed for lipase activity and total protein content (A280).

The fractions showing lipase activity were pooled. The specific activity of the purified enzyme was compared with that of the initial crude enzyme and the purification factor was calculated. The active fractions were stored at 4°C until used for polyacrylamide gel electrophoresis.

Biochemical and kinetic characterization of partially purified lipase

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and activity staining. SDS- Poly acryl amide Gel Electrophoresis of proteins was carried out on a vertical slab midi-gel apparatus at 40-80 mA to establish the purity of the lipase protein. The SDS-PAGE was prepared. The enzyme solution diluted with electrophoresis sample buffer (0.6 g Tris, 8 mL glycerol and 14 mL water, pH was adjusted at 6.75 with con. HCl 0.16 g SDS, 4 mL 2-mercaptoethanol and 0.002 g Bromophenol blue to 20 mL with D/W) was boiled for 5 min on a water bath.

The SDS-PAGE was performed on 12% polyacrylamide gel (with 5% stacking gel) and the relative molecular mass of proteins was determined with reference to Perfect protein markers (10.0-225 kDa, Novagen, India). The 50KDa band is a high intensity reference band. After migration, the gels were staining with Coomassie Brilliant Blue R-250.

The gel was stained by silver staining method. The gel stained with Coomassie Brilliant Blue stain was kept in destaining solution (50mL methanol, 10mL acetic acid and 40mL water), which acts as

fixative. The gel was allowed to destain for 30 minutes. After removing the excess of fixing solution the gel was transferred in 50% methanol for 20 minutes. This step was repeated two times. Thereafter the gel was washed twice with distilled water for 5 minutes. It was then transferred to 0.02% sodium thiosulphate for 1 minute. Again the gel was washed twice with distilled water for 2 minutes. The gel was then immersed in 0.2% silver nitrate solution for 30 minutes. After silver nitrate treatment, the gel was washed twice with distilled water for 1 minute. The gel was then immersed in developing solution (6g Na_2CO_3, 80 mL D/W and 20 ml 0.02% Sodium thiosulphate.) until the gel was stained and developing the bands. The development was stopped by addition of stopping solution (10% acetic acid). A dark yellow colour appeared within 30 min.

Effect of substrate concentration on lipase activity

The affinity of the lipase towards substrate (pNPP) was studied in detail. Assays with lipase were performed in Phosphate buffer, pH 8.0 at 40°C with increasing concentrations of substrates from 2 μM to 30 μM/mL.

Effect of temperature on activity and stability of lipase

The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures ranging from 10-80 °C with 10°C differences. Lipase activity was assayed under standard assay conditions.

The thermo stability of the lipase fraction was studied by incubating the enzyme extract at various temperatures (10, 20, 30, 40, 50, 60, 70 and 80°C) in a water bath for 15, 30, 45 and 60 min. At the end of the incubation period, the enzyme extract was rapidly cooled and the remaining lipase activity was assayed.

Effect of pH on activity and stability of lipase

The lipase was assayed at different pH (6-13, with 1.0 unit difference) values. Phosphate buffer was used for pH 6, 7 and 8. Glycine buffer was used for pH 9, 10, 11, 12 and 13. Reactions were stopped after incubation, and lipase activity was assayed under standard assay conditions.

The effect of pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 6.0 to 13.0 for 30 min at 40°C in a water bath. Reactions were stopped after incubation, and the residual lipase activity was assayed.

Effect of metal ions on lipase activity

The effects of various divalent metal-ions, namely Ca^{++}, Co^{++}, Cu^{++}, Pb^{++}, Hg^{++}, Mg^{+}, Mn^{++} and Zn^{++} as $CaCl_2$, $Co(NO)_3$, $CuSO_4$, $Pb(NO_3)_2$, $Pb(NO_3)_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$, monovalent ions, namely Na^+ and K^+ as their chloride salts (KCl and NaCl, respectively) and EDTA on lipase activity were studied.

Different concentrations of 1mM, 3mM and 5mM were checked. Enzyme were incubated with equal volumes of the various metal ions and incubated for 1 h at 40°C. Relative activity of lipase after incubation period was assayed.

RESULTS AND DISCUSSION

Purification of *Saccharomonospora azurea* AP11/18 lipase

An extracellular lipase produced by *Saccharomonospora azurea* AP11/18 was purified by employing a three-step procedure, namely precipitation, dialysis and gel exclusion chromatography. The results of the purification profile are summarized in Table 6.1. For substrate affinity and kinetic characterization pNPP was employed as a substrate in the lipolytic assay, and assay was carried out under standard condition. (Haas and Joerger, 1995).

Ammonium sulphate precipitation

The enzyme produced after optimization of the cultural conditions was subjected to ammonium sulphate precipitation for salting out the proteins. 100 mL of the crude fermented broth were used after 96 h of fermentation (19.64 U/mL, 2.254 mg protein/mL) of *Saccharomonospora azurea* AP11/18. The culture broth was filtered and the filtrate was precipitated with 80% (w/v) $(NH_4)_2SO_4$. Residues collected after vacuum filtration was dissolved in 0.2 M phosphate buffer. A considerable increase in the specific activity was observed. The specific activity of concentrated protein was 25.76 U/mg of protein as compared to 8.73 U/mg of protein for the crude enzyme extract. This step yielded 15.48% recovery and 2.95 times fold purification shown in Table 1.

Table 1. Summary of Lipase purification steps

No.	Fraction	Total volume mL	Enzyme Activity U/mL /min	Protein in mg/mL	Total Enzyme Activity U/mL/min	Total Protein mg/mL	Specific Activity U/mg	Purification fold	% Recovery
1.	Crude Enzyme	100	19.69	2.254	1969	225.4	8.73	1	100
2.	Precipitation with Ultra filtration	10	30.48	1.183	304.85	11.83	25.76	2.95	15.48
3.	Dialysis	10	30.99	0.483	309.9	4.83	64.16	7.34	15.73
4.	Gel Filtration Chromatography	5	80.17	0.214	400.83	1.07	374.62	42.91	20.35

Dialysis of precipitated enzyme

The enzyme precipitate was dialysed to concentrate the purified enzyme as well as for desalting of excess ammonium sulphate. Dialysis yields 15.73% recovery with 7.34 fold purification, having a specific activity of 64.16 U/mg of protein.

Gel Filtration chromatography

Shephacyl HR-200, is a cross-linked polymer used for gel filtration chromatography, which is used for differentiating the molecular size. The dialyzed protein containing enzyme was subjected to Shephacyl HR-200 for separation of lipases from other proteins. The representation of optical density for all the fractions measured at 280 nm in UV-Vis spectrophotometer is given in Fig 1. Each fractions showing greater than 0.1 O.D at 280nm were subjected to lipase assay. Lipase activity of each fraction is given in Fig. 2.

The column bound proteins were eluted as one major peak (Fig. 1). But 2 peaks observed in lipase activity, one is minor and other is major indicating the presence of more than one component (Fig. 2). The fractions containing high amount of protein exactly coincide with the high enzyme containing fractions.

The fractions showing high lipase activity were pooled and enzyme kinetics studies were performed with them. The specific activity after this step of purification was 374.62 U/mg of specific activity with 42.91 times fold purification and 20.35% recovery.

Biochemical and Kinetic characterization of partially purified Lipase

Molecular weight determination by sodium dodecyl sulphate polyacrylamide gel electrophoresis

A molecular mass of approx 50 and 42 kDa was determined for the purified lipase by SDS-PAGE (Fig. 3). We observed the band of partially purified Lipase on SDS PAGE gel near the marker band of 42kDa and 50kDa. Two protein bands were observed after Sephacyl HR 200 chromatography.

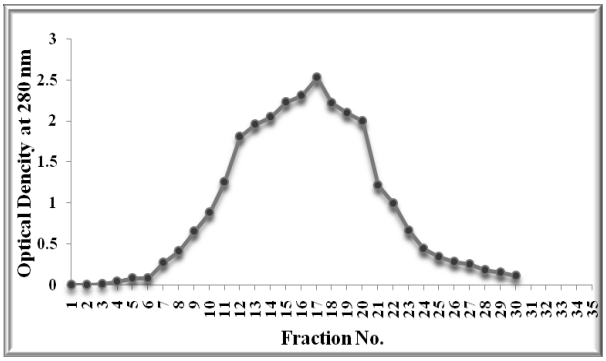

Fig. 1 Optical density plot for the fractions obtained after gel filtration chromatography

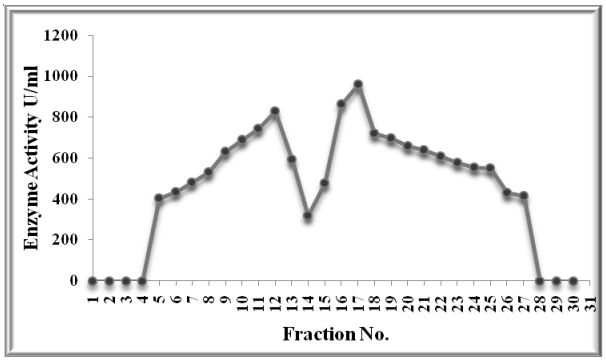

Fig. 2 Lipase activity plot for the fractions obtained after gel filtration chromatography

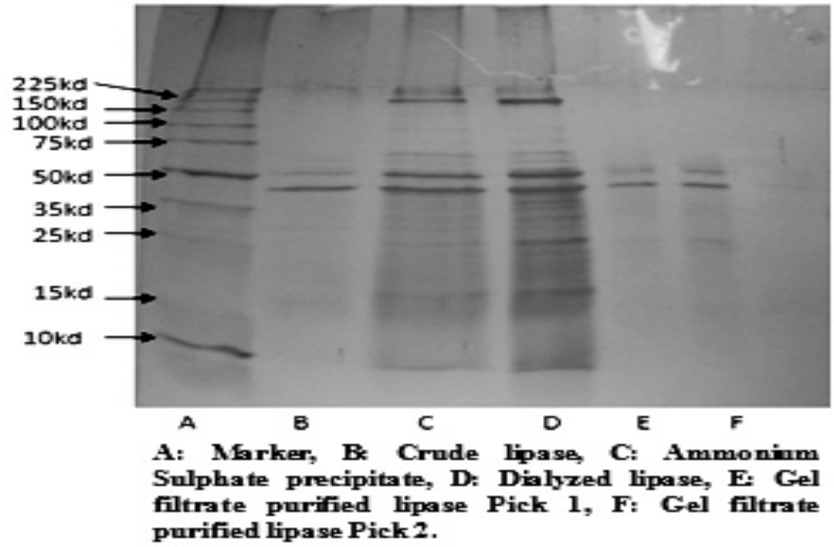

Fig. 3 Separation of lipase-containing fractions by SDS-PAGE

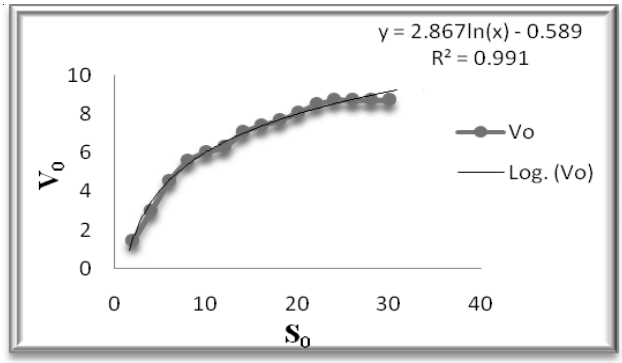

Fig. 4 Effect of substrate concentration on lipase activity.

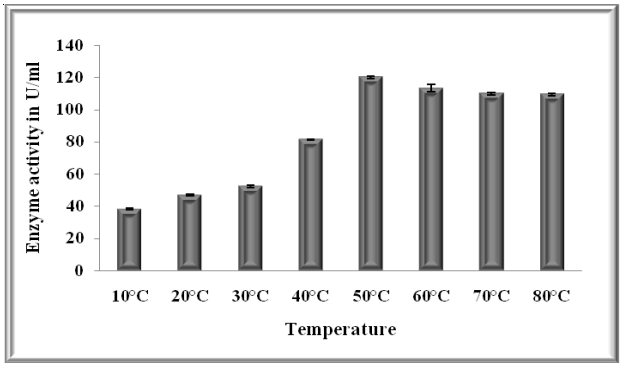

Fig. 6 Effect of Temperature on lipase activity

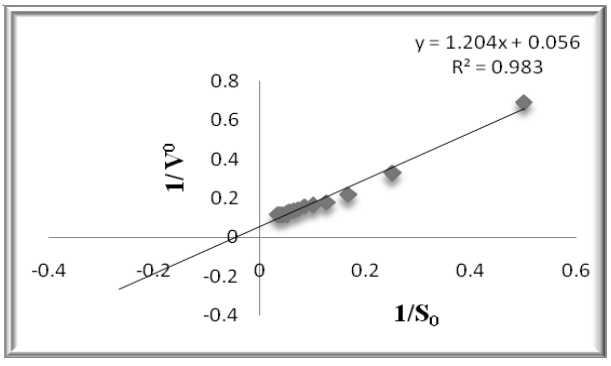

Fig. 5 Line weaver Burk plot for Lipase activity (Km 21.5 μMoles and Vmax was 17.85 U/mg)

These two bands were dominant to see in all purifying steps. Which confirms that lipase is made up of two polypeptide chain having molecular weight 50kDa and 42kDa. Among these two 42kda is more dominant over 50kDa. These finding is generally in agreement with those reported in the literature for molecular weight of lipase.

Iwai *et al*., (1975) purified two lipases from *Penicillium cyclopium* having MW of 27 kDa and 36 kDa, while Aloulou *et al*., (2007) reported 38.48 kDa MW containing lipase from Yarrowia lipolitica. The molecular weight of *Pseudomonas aeruginosa* BN-1lipase on SDS-PAGE was found to be approximately 60.0 kD (Syed *et al*., 2010).

Effect of substrate concentration on Lipase activity

To study the enzyme-substrate affinity, the kinetic parameters of the different substrates concentration were determined from the purified fractions. The activity increased progressively till 24 μMoles and there was no further increase

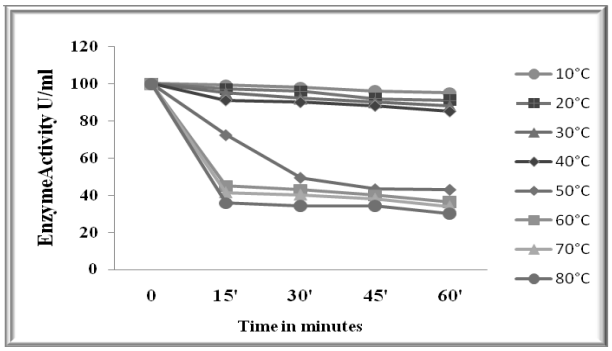

Fig. 7 Effect of thermal stability on lipase activity

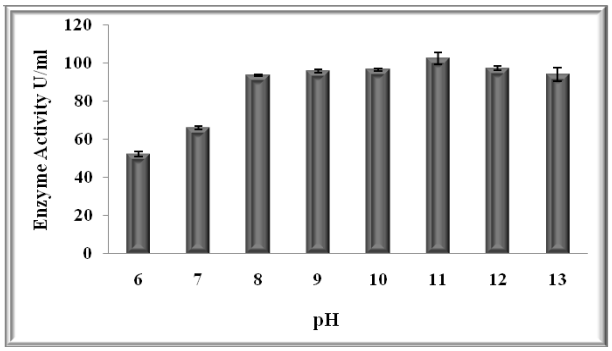

Fig. 8 Effect of pH on lipase activity

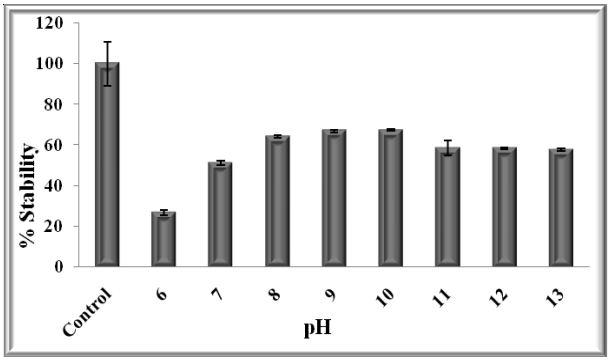

Fig. 9 Effect of pH stability on lipase activity

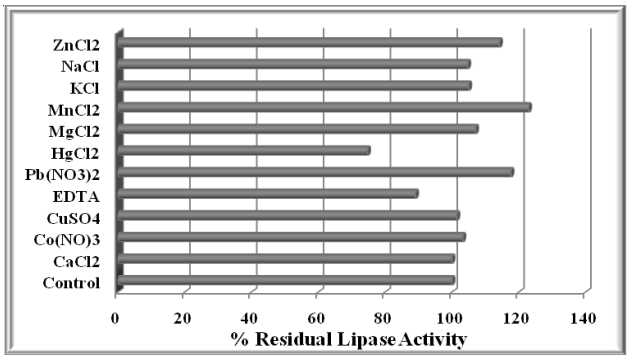

Fig. 10 Effect of metal ions (3mM) on lipase activity

observed. The Km calculated for pNPP by Line weaver Burk plot is 21.5µMoles and Vmax was 17.85 U/mg. The results are depicted in Fig. 4 and 5. This is the first report of lipase production from *Saccharomonospora azurea*.

Effect of Temperature on activity and stability of lipase

Lipase is known to be a thermos table enzyme which has optimum activity at high temperature. *Saccharomonospora azurea* AP11/18 was observed at 50°C (Fig. 6). There was good enough activity observed in the range of 40 to 80°C. This type of broad range is beneficial for industrial purposes because maintenance of narrow range is very difficult at commercial level. Bacterial lipase has been reported with an optimum temperature within a range of 30-60°C.

To study the thermal stability of lipase enzyme, the enzyme extract was incubated at different ranges of temperature, i.e., from 10-80°C. the results (Fig. 7) shows that lipase retain 95% activity at 10 to 40°C up to half one hour of incubation, while it retain 70% at 50°C and 45 to 35% its activity between 60 to 80°C. Further increase in the incubation temperature, the activity of the enzyme was inhibited. At the end of one hour, 90 to 95% activity was retained at 10 to 40°C and 30 to 40% retained at 50 to 80°C. It might be due to the denaturation of the enzyme at high temperature.

Effect of pH on activity and stability of lipase

The optimum pH range observed for lipase produced by *Saccharomonospora azurea* AP 11/18 was from 6 to 13. The optimum activity was observed at 11.0 pH and inactivated at pH 5 therefore it can be characterized as an alkalophilic enzyme (Fig. 8). A comprehensive review of all bacterial lipase done by Gupta, *et al.* 2004, stated that maximum activity of lipase at pH value higher than 7.0 has been observed in many cases. Bacterial lipases have a neutra or alkaline optimum pH with the exception of P.fluorenscnes that has an acidic optimum pH at 4.8. But in general bacterial lipases are stable in a wide range of pH, from 4 to 11.

To check the stability of lipase, purified enzyme was incubated in various pH buffer for 30 min. *Saccharomonospora azurea* AP 11/18 lipase could tolerate a pH range of 6.0-13.0 Lipase retain 60 to 65% of its activity at 8 to 10 pH and 50 to 55% its activity at 7 and 11 to 13pH, while retain 20 % of its

activity at 6 pH. The optimum lipase stability was observed at pH 10 (Fig. 9). The remarkable stability of *Saccharomonospora azurea* AP 11/18 lipase in this range has proved it to be a potential alkaline lipase. The stability of the lipase decreased sharply after 30min. of incubation. This indicates that *Saccharomonospora azurea* AP 11/18 lipase is a thermophilic enzyme.

Effect of metal ions on lipase activity

The effect of various ions at the concentration of 1mM, 3mM and 5mM was checked on Lipase activity. After one hour incubation of pure lipase with metal ion (1mM, 3mM and 5mM), lipase activity was checked. Among 3 different concentrations, 3mM concentration gives remarkable difference in lipase activity among the metal ions. Manganese is the core activator of lipase (Fig. 10). It increases 22.93% of lipase activity. The Next to that lead and Zinc are the next known activators of the *Saccharomonospora azurea* lipase and enhanced the activity of lipase 17.62% and 14.2 % respectively. Magnesium was also found to activate the lipase enzyme to some extent (7.06%). Sodium, Potassium Cobalt and calcium had little effect on lipase activity. Mercury and EDTA act as inhibitor of the lipase activity. The results are depicted in Fig. 10.

CONCLUSION

The results from our present study have demonstrated the occurrence of an extracellular lipase, with molecular weight of 50 and 42 kDa derived from cultures of *Saccharomonospora azurea* AP 11/18. The present study showed unique properties of an extra-cellular alkaline metallolipase of *Saccharomonospora azurea* AP 11/18 purified by precipitation and gel exclusion chromatography. The purified lipase showed good thermostability and had optimums of activity at 50°C and alkaline pH (pH 8.0). The Km and Vmax value for pNPP is 21.5µMoles and 17.85 U/mg, calculated by Line weaver Burk plot. Of the metal ions tested, Manganese is the core activator of lipase. Lead and Zinc are the next known activators of the *Saccharomonospora azurea* lipase. Sodium, Potassium, Cobalt and calcium had little effect on lipase activity. Mercury and EDTA act as inhibitor of the lipase activity. Alkaline thermostable nature of Saccharomonospora azurea lipase makes it highly potent for future biotechnological applications.

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