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Characterization of a thermotolerant purified lipase of *Bacillus licheniformis* MTCC-10498

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ABSTRACT

An alkaline thermotolerant lipase from *Bacillus licheniformis* MTCC-10498 (purified by the techniques of ammonium sulphate precipitation, dialysis and hydrophobic chromatography) was characterized for reaction pH, reaction temperature, thermostabilty, substrate specificity and other parameters etc. The purified lipase (2.0 U/ml; protein content 1.05 mg and specific activity 31 U/mg) the optimal activity of purified lipase was seen at pH 8.0 and at temperature of 55 ºC (2.0). The hydrolytic activity of purified lipase preparation reduced to approx. 50%, in 3h. The presence of metal salts and EDTA inhibited lipase activity of purified lipase. Presence of PMSF (20mM) in the reaction mixture severely decreased the hydrolytic activities.

***Keywords*: *Bacillus licheniformis*,** lipase, hydrolytic activity, pH, temperature, EDTA.

1. INTRODUCTION

Lipases have emerged as key enzymes in various industries like food, chemical, pharmaceutical, cosmetic and detergent production and leather processing (Pandey et al., 1999). Lipases of microbial origin have been explored to catalyze the synthesis of variety of esters of sugars and carbohydrates. In recent years there has been a great demand for thermostable enzymes in industries. The necessity of thermostable lipases for different applications has been growing rapidly (Chen et al., 2007; Shah et al., 2007 Sharma et al., 2011; Sharma et al., 2012). Thermostable enzymes are active and stable at temperature higher than optimal growth of their producer strains (Saboto et al.,1999; Mozersky et al., 2002 Sharma et al., 2011). The standardization of pH, temperature, thermal stability, substrate specificity is necessary. The application of thermostable lipases in fatty acid/carbohydrate esters are of immense use as nutraceuticals and additives in cosmetics (Sharma et al., 2012) They also appear to possess surfactant properties that must be explored. Lipases catalyzes both esterification as well as transesterification reactions in organic free/ restricted media.

2. MATERIALS AND METHODS

2.1 Chemicals

$NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, $FeSO_4.{}_7H_2O$, $(NH_4)_2SO_4$; yeast extract and gum acacia (Hi-Media Laboratory, Ltd., Mumbai, India); sucrose, KCl, KI, KNO_3, isopropanol, ammonium persulphate, 2-mercaptoethanol, HCl, p-nitrophenyl formate (p-NPF), p-nitrophenyl acetate (p-NPA), p-nitrophenyl benzoate (p-NPB) p-nitrophenyl caprylate (p-NPC), p-nitrophenyl laurate(p-NPL), p-nitrophenyl palmitate (p-NPP) from Alpha-aesar, Heysham, England. Acetic acid and Triton–X100, Tween-20, 40 and 80 (Qualigens Chemicals, Mumbai, India); phenyl methylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), sodioum lauryl sarcosine (SLS), acrylamide, bisacryl amide (N,N-methylenebisacrylamide) glycerol, glycine and Tris (2-

hyroxymethyl-2-methyl-1,3-propanediol) (Sigma Chemicals Co., USA). All chemicals were of analytical grade and were used as received.

2.2 Assay method for lipase and protein estimation

The lipase activity was assayed using p-nitrophenyl palmitate (p-NPP), a chromomeric substrate. Lipase activity of crude lipase, purified or matrix-bound lipase was assayed employing a modified colorimetric method (Winkler and Stuckmann, 1979). The stock solution (20 mM) of p-NPP was prepared in HPLC grade isopropanol. The reaction mixture contained 80 µl of p-NPP stock-solution, 20 µl of lipase and Tris buffer (0.05 M, pH 8.5) to make final volume 3 ml. The reaction mixture was incubated at 55 °C for 10 min in a water bath (Banglore Genei Pvt. Ltd., Banglore). Further lipase reaction was stopped by chilling at -20 °C for 5 min. A control containing heat-inactivated (5 min in boiling water bath) enzyme was also incubated with each assay. The absorbance (A_{410}) of heat-inactivated lipase was subtracted from the absorbance of the corresponding test sample. The absorbance A_{410} of the p-nitrophenol released was measured and expressed in millimoles (mM). The unknown concentration of p-nitrophenol released was determined from a reference curve of p-nitrophenol (2-20 µg/ml in 0.05 M Tris buffer pH 8.0). Each of the assays was carried out in triplicate. Stock solutions of various p-nitrophenyl esters p-NPF, p-NPA, p-NPC and p-NPL were also prepared for use in the some of the experiments.

2.3 Purification of *B. licheniformis* MTCC-10498 lipase

The purification of bacterial lipase was performed using techniques of ammonium sulphate salting out, dialysis and hydrophobic interaction chromatography (Octyl sepharose) respectively (Sharma et al., 2012).

2.3.1 Effect of buffer pH on lipase activity

To determine the optimum pH for lipase assay, pH of assay buffer (Tris buffer 0.05 mM) was set at 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0 10.5 and 11.0. Lipase activity was then measured. To the reaction mixture containing p-NPP and Tris-buffer at each of the above pH, 20 µl of purified lipase was added and the reaction was carried out at 55 ºC for 10 min.

2.3.2 Effect of reaction temperature on lipase activity

Next experiment was done to optimize the reaction temperature for lipase assay. For this purpose the reaction buffers were incubated at 45, 50, 55, 60 and 65˚C.

2.3.3 Effect of reaction time on lipase activity

To determine the optimum reaction time, the reaction mixture incubated at 55˚C was incubated for varying time range of 5, 10, 15 and 20 min followed by lipase assay.

2.3.4 Determination of substrate specificity of lipase production

The substrate specificity was determined by using a variety of p–nitrophenyl esters *viz*. p–NP formate, p-NP acetate, p–NP benzoate, p–NP myristate, p–NP caprylate, p-NP laurate and p-NP palmitate prepared as 20 mM stocks in isopropanol. The amount of p–nitrophenol released was assayed spectrophotometrically at A_{410}.

2.3.5 Thermo-stability of lipase

To examine the effect of the reaction temperature on stability of the enzyme, purified enzyme, purified enzyme, matrix bound enzyme (0.2 ml) was kept separately in eppendorf tubes up to 5 h at and 55 ºC. At intervals (1 h) 20 µl of purified lipase was sampled and assayed for residual lipase activity. The activity measured immediately before incubation was defined as 100% of hydrolytic activity.

2.3.6 Effect of salt-ions on lipase activity

To evaluate the effect of various salt- ions on lipase activity, an attempt was made to study the effect of salt ions (Li^+, Na^+, K^+, Fe^{3+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Cu^{2+} as $LiCl^3$, NaCl, KCl, $FeCl_3$, $ZnSO_4$, $MnCl_2$, $CoCl_2$, $HgCl_2$, $MgCl_2$ and $CuSO_4$ respectively) on lipase activity. Each of the salt ions was separately, included in the reaction mixture at a final concentration of 1 mM. The lipase activity was assayed after 10 min of incubation at 55 ºC.

2.3.7 Effect of EDTA (chelating agent) and surfactants

EDTA stock at the concentration of 50, 100, 150 and 200 mM was separately added in the each of the reaction mixture tube and lipase activity was checked thereafter. Various detergents both ionic as well as non-ionic have the property to denature proteins. To study the effect of such detergents on *B. licheniformis* lipase, each detergent (Tween-20, Tween-60, Tween-80,

Triton X-100, Sodium dodecyl sulphate and Sodium lauryl sarcosine; 0.05%, v/v) was included separately in reaction mixture. A lipase activity was recorded at A_{410} after 10 min incubation at 55 ºC.

2.3.8 Effect of PMSF on lipase activity

A serine protease inhibitor PMSF was included in the reaction mixture at an interval of 5, 10, 15 and 20 min of initiation of lipase reaction. Kinetics of inhibition was studied at a set concentration of PMSF (1, 5 and 10 mM) in lipase reaction cocktail incubated at 55 ºC.

3. RESULTS AND DISCUSSION

3.1 Characterization of *B. licheniformis* MTCC-10498 lipase

Purified lipase of *B. licheniformis* MTCC-10498 was characterized for various physico-chemical parameters with appropriate optimal enzyme content.

3.2 Effect of pH on purified

Actual inorganic state of lipase was essential for its optimal activity. The optimal activity of *B. licheniformis* MTCC-10498 purified lipase was seen at pH 8.0 (2.0 ± 0.01). The lipase activity decreased more sharply at acidic pH. The lipase from *B. licheniformis* MTCC-10498 was active over a wide pH range (4-11). Earlier a marine strain (*B. licheniformis*) lipase was also active over a wide pH range (Lowry et al., 1951).

Table 1: Effect of pH on purified lipase activity

pH	Purified lipase (U/ml)
4.0	**0.150 ± 0.01**
4.5	0.236 ± 0.03
5.0	0.642 ± 0.01
5.5	0.705 ± 0.02
6.0	0.866 ± 0.02
6.5	0.978 ± 0.01
7.0	1.162 ± 0.03
7.5	1.734 ± 0.02
8.0	**2.000 ± 000**
8.5	1.779 ± 0.01
9.0	1.762 ± 0.02
9.5	1.552 ± 0.02
10.0	1.168 ± 0.04
10.5	0.814 ± 0.02
11.0	0.315 ± 0.02

3.3 Effect of temperature on purified lipase activity

The purified lipase exhibited maximum lipase activity at pH 8.0 *viz.* 2.0 ± 0.01 at 40-70 ºC (Table 2). The optimum temperature for enzyme activity was 55 ºC and it was reported high in an earlier study with the lipase of *Pseudomonas* sp. (Annamali et al., 2011).

Table 2: Effect of temperature on lipase activity

Temperature	Purified lipase (U/ml)
40	0.864 ± 0.01
45	1.36^{9} ± 0.01
50	1.804 ± 0.02
55	**2.000 ± 0.01**
60	0.988 ± 0.02
65	.612 ± 0.01
70	0.468 ± 0.05

3.4 Substrate specificity of purified lipase

The purified enzyme exhibited highest affinity towards the p-NPP followed by p-NPM. The purified lipase was specific toward p-NPP but shows variable specificities towards other esters. The hydrolytic activity of purified lipase for hydrolysis of p-NPM was approximately lower than p-NPP. Hydrolytic activity for p-NPF for purified lipase was 14.6% lower than for p-NPP (Table 3). The bacterial lipase thus showed a preferentially higher specificity and hydrolytic activity towards the longer C-chain esters.

However, it appeared that p-NPP was hydrolyzed most efficiently than the other selected acyl esters. In a previous study p-NPP was most favourable substrate by *B. coagulans* lipase (Sughihara et al., 1991).

Table 3: Substrate specificity of purified

pNP-Substrate(20 mM)	Purified lipase (U/ml)
Formate (C1)	0.292 ± 0.01
Acetate (C2)	0.362 ± 0.01
Benzoate (C6)	0.582 ± 0.01
Caprylate (C8)	0.982 ± 0.04
Laurate (C12)	1.672 ± 0.02
Myristate (C14)	1.718 ± 0.01
Palmitate (C16)	**2.000 ± 0.030**

3.5 Thermostability of purified lipase

The hydrolytic activity of purified lipase (0.69 ± 0.01) preparation reduced to approx. 50%, in 3 h. After 5 h lipase activity reduced to 22% in case of purified lipase (Table 4). Lipase from *B. licheniformis* shown to have good thermostability at 55 ºC (Sinchaikul et al., 2001; Dheeman et al., 2010) but decreases sharply after 60 ºC.

Table 4: Thermostability of lipase

Time (h)	Purified lipase (U/ml)
0.5	1.872 ± 0.05
1.0	1.603 ± 0.04
2.0	1.291 ± 0.06
3.0	0.923 ± 0.05
4.0	0.692 ± 0.01
5.0	0.413 ± 0.07
Control	**2.000 ± 0.03**

3.6 Effect salts on lipase activity of purified lipase

The presence of metal salts of Li^{3+}, Na^{+}, K^{+}, Fe^{3+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Hg^{2+}, $Mg^{2+}$and Cu^{2+} inhibited lipase activity of purified lipase (Table 7). In a previous study, Fe^{3+}, Mn^{2+} declined the enzyme activity (Sekhon et al., 2005; Tewari et al., 2011).

Table 5: Effect of salt ions on lipase activity

Salts (1 mM)	Purified lipase (U/ml)
Control	2.000 ± 0.03
$LiCl_3$	1.500 ± 0.05
NaCl	1.090 ± 0.04
KCl	1.167 ± 0.04
$FeCl_3$	1.876 ± 0.02
$ZnSO_4$	1.416 ± 0.03
$MnCl_2$	1.576 ± 0.02
$CoCl_2$	1.236 ± 0.01
$HgCl_2$	1.161 ± 0.04
$MgCl_2$	1.067 ± 0.03
$CuSO_4$	1.678 ± 0.02

3.7 Effect of EDTA (chelating agent) and surfactants

It was observed that as the concentration of EDTA increased activity decreased drastically. The free enzyme has left residual activity of 23% (Table 6). The activity of the enzyme was inhibited by EDTA (Gao et al., 2000; Etaweel et al., 2005; Bancerz and Ginalska, 2007).

Table 6: Effect of EDTA (chelating agent)

EDTA (mM)	Purified lipase (U/ml)
50	1.567 ± 0.03
100	0.962 ± 0.04
150	0.812 ± 0.02
200	0.468 ± 0.04
Control	2.0 ± 0.03

Except for Tween-80 (0.05% v/v) all other detergents reduced the activity of purified lipase (Table 7). Lipase activity of purified lipase remains same with the treatment of Tween-80. Similarly, Triton X-100 inhibited activity of the lipase from *Mucor* sp. (Abbas et al., 2002) and *Rhizopus* sp. (Sroka, 1994). The concentration of Tween 80 (v/v) was studied and a conc. of 0.05% was optimal. A slight increase or decrease in concentration of Tween-80 may result in decreased activity (Table 8).

Table 7: Effect of detergents on lipase activity

Surfactant (0.05%, v/v)	Purified lipase (U/ml)
Control	2.000 ± 0.03
Tween-20	1.800 ± 0.03
Tween-60	1.868 ± 0.04
Tween-80	**2.000 ± 0.02**
Triton-X100	0.996 ± 0.02
SDS	0.918 ± 0.03
SLS (Sodium lauryl sarcosine)	0.816 ± 0.04

Table 8: Effect of tween-80 concentration

Surfactant Tween-80 **(%, v/v)**	**Purified lipase (U/ml)**
Control	2.000 ± 0.03
0.04	1.842 ± 0.01
0.05	**2.000 ± 0.02**
0.06	1.749 ± 0.01
0.07	1.612 ± 0.02
0.08	0.816 ± 0.04

3.8 Effect of PMSF on lipase activity of purified free lipase

Inhibition kinetics of *B. licheniformis* MTCC-10498 lipase was directly proportional to PMSF concentration (i.e., at 1, 5, 10 and 20 mM). The activity of lipase declined (Table 9). Presence of PMSF (20mM) in the reaction mixture severely decreased the hydrolytic activities in an earlier study lipase activity reduced at 70mM (Chakraborty and Raj, 2008).

Table 9: Effect of PMSF on lipase activity

PMSF (Concentration)	5 min	10 min	15 min
None	1.203 ± 0.02	2.000 ± 0.03	1.976 ± 0.02
5 mM	1.612 ± 0.01	0.892 ± 0.04	0.814 ± 0.01
10 mM	0.868 ± 0.02	0.742 ± 0.03	0.256 ± 0.05
15 mM	0.420 ± 0.03	0.202 ± 0.03	0.256 ± 0.05
20 mM	0.030 ± 0.001	0.040 ± 0.002	0 .070 ± 0.001

3.9 Kinetic behaviour of purified lipase of *B. licheniformis* MTCC-10498 lipase

Substrate specificity and effect of concentration of p-NPP on the reaction rate catalyzed by this lipase was studied. A Lineweaver Burk plot was linear at low concentration for p-NPP (Fig. 1). The *K*m is recorded as 32.33 mM and *V*max was observed as 4.71 µmole/ min/ mg protein.

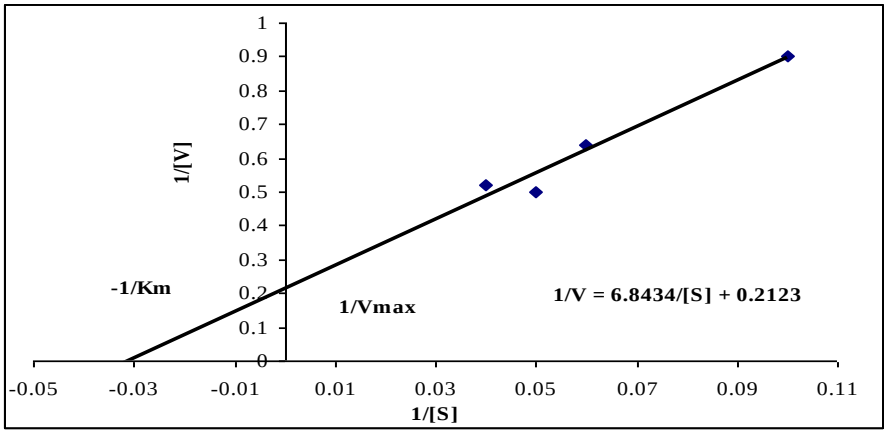

Fig. 1: Lineweaver-burk plot for reaction catalyzed by purified lipase of *B. licheniformis* MTCC-10498

4. CONCLUSION

In the present study, the lipase produced by *B. licheniformis* isolated from hotspring water, was cost effective because of cheaper source as cottonseed oil, stable at 55°C and active over a wide pH range (4-11). Hence it could be useful for enzyme industry.

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