Purification and Physicochemical Properties of Lipase from Thermophilic *Bacillus aerius*

Nitin Kumar Saun, Poonam Mehta and Reena Gupta*

Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-171005, India

Abstract: A thermophilic bacterial isolate producing lipase was isolated from soil of hot spring and identified as *Bacillus aerius* (MTCC 10978). Peak lipase activity was observed when 30 h old inoculum was used and incubated in shaking conditions for 48 h. The optimal temperature and pH for the bacterial growth and lipase production was found to be 55°C and 8.0 respectively with cottonseed oil as carbon source, yeast extract and beef extract as nitrogen source. The enzyme produced by thermophilic *Bacillus aerius* (MTCC 10978) was purified to 9-fold with 7.2% recovery by ammonium sulfate precipitation and DEAE-Cellulose Column Chromatography. The enzyme was found to be a protein having a molecular weight of 33 kDa on SDS-PAGE. The *K*<sub>m</sub> and *V*<sub>max</sub> value of lipase using *p*-nitrophenyl palmitate as calculated from Lineweaver-Burk plot was 2.13 mM and 0.66 μmol/ml/min respectively.

Key words: *Bacillus aerius*, lipase, extracellular, thermostable and purification

1 Introduction

Lipases (triacylglycerol acyl ester hydrolases; EC 3.1.1.3) find prominent position as industrial biocatalysts<sup>1</sup>). They catalyze the hydrolysis of triglycerides at water oil inter phase to yield free fatty acids, monoglycerides, diglycerides and glycerols<sup>2, 3</sup>). Lipases are ubiquitous in nature and are produced by various plants, animals and micro-organisms. The ease, with which enzymes could be isolated from microbes, has made bacteria predominant sources of lipase. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology as they can be mass cultured and genetically modified easily<sup>4</sup>). Lipases are versatile biocatalysts showing multiple applications in a wide range of biotechnological processes. Also, lipases do not require cofactors to catalyse hydrolytic reactions and remain active in the presence of organic solvents<sup>5</sup>). Bacterial lipases are produced by both submerged and solid-state fermentation<sup>6, 7</sup>). Lipase production requires carbon and nitrogen sources as required by any fermentation process. Most of the lipase production studies do not use simple sugars as carbon sources rather use lipid substrates as sole carbon source<sup>8</sup>). Thermostable enzymes find wide applications in industry because of their high activities at elevated temperatures and stabilities in organic solvents<sup>9</sup>). Moreover the activity of enzyme is stabilized by various immobilized techniques with inert supports, also its thermostability and usability can be enhanced by immobilization onto hydrophobic support like nylon 6, molecular sieve etc<sup>10, 11</sup>). Most of the microbial lipases are active at alkaline pH (pH 7.0-11.0). Any alteration in pH of the reaction mixture is likely to affect the catalytic activity of lipases. Studies on thermostable lipases from *Bacillus* sp. that are active at alkaline pH have been reported previously from our lab<sup>12</sup>). These enzymes have been used for synthesis of various esters<sup>13, 14</sup>). For newly isolated microbes, it is essential to characterize the enzyme if it is intended to be used in biotechnological application(s). This is the first report of extracellular, thermostable and alkaline lipase being produced and purified from *Bacillus aerius*. Its unique properties with respect to specificity, stability, temperature and pH dependence have been studied for possible application in synthesis of esters with anti-cancerous properties.

2 Materials and methods

2.1 Biological material

The lipase producing bacteria were isolated from the soil and water samples of a hot-spring named Tattapani, Kullu and sweet shops around Shimla. The thermophilic *Bacillus aerius* (identified at IMTECH, Chandigarh) was grown in the medium of following composition; yeast extract (2 g/L), peptone (5.0 g/L), sodium chloride (5.0 g/L), beef extract...
(1.5 g/L), ammonium chloride (1.0 g/L) and cottonseed oil (10 mL/L; emulsified with 0.5% Gum Acacia) at pH 8.0. The seed culture (7.5% inoculum) was transferred to 50 ml production medium (250 ml Erlenmeyer flask) for 48 h under shaking conditions at 110 rpm at 55°C. The culture broth was centrifuged at 10,000 rpm for 10 min at 4°C. The lipase activity was assayed both in the supernatant as well as in pellet for determining extracellular and intracellular enzyme activity respectively.

2.2 Assay of lipase enzyme:

Lipase activity was measured by colorimetric method of Winkler and Stuckmann by using p-nitrophenyl palmitate as a substrate. To 2.9 ml of Tris-HCl buffer (0.1 M, pH 9.5) emulsified with gum acacia (0.1% w/v) was added 80 µl of the substrate (p-nitrophenyl palmitate, 20 mM) which was incubated at 55°C for 10 min and 20 µl of enzyme was added thereafter. The reaction mixture was again incubated at 55°C in water bath for 10 min. The reaction was stopped by chilling at ~80°C for 4-5 minutes. The amount of p-nitrophenol released was measured at 410 nm. A standard curve of p-nitrophenol was plotted (concentration 10-100 µg/ml). By recording the absorbance at 410 nm of test sample, the corresponding concentration of p-nitrophenol was determined from reference curve. Each of the experiments were performed in triplicates and mean values ± Standard Deviation (SD) were presented. Statistical analysis was performed on SPSS version 20. A p value <0.05 was considered as significant.

2.3 Lipase activity

One unit of lipase activity was defined as amount of enzyme required to release one micromole of p-nitrophenol from the substrate (p-nitrophenyl palmitate) per minute per ml of the enzyme preparation under standard assay conditions.

3 Optimization of culture conditions for maximum lipase production

To optimize the inoculum age and size for the enzyme production, production medium (nutrient broth, pH 8.0) was inoculated with inocula of varying age, 6 to 48 h and inoculum size between 2.5% to 15.0%. The supernatant was assayed for lipase activity. Under above optimized inoculum age and size, the production medium was incubated in shaker for the time intervals of 6 to 72 h. To determine the optimum temperature and pH, the lipase production was studied at temperature range of 30°C to 65°C and pH 6.0 to 9.5. Different carbon sources (1%) viz.: cottonseed oil, mustard oil, olive oil, coconut oil and soyabean oil, emulsified with 0.5% (w/v) gum acacia were supplemented in the production medium. The carbon source resulting in maximum enzyme production was added to the production medium in varying concentrations of 0.5%, to 2.5% (w/v). The lipase activity was determined in the cell free supernatant of the culture broth. Similarly, each of the various nitrogen sources viz. beef extract, gelatin, urea, ammonium chloride, peptone, potassium nitrate and yeast extract was individually added to the production at concentration of 0.5% (w/v). The nitrogen source resulting in the maximum enzyme production was added in varying concentrations of 0.1% to 0.5% (w/v). The growth profile of the microorganism was studied by withdrawing the samples from the culture flasks at regular intervals from 6 h upto 96 h. The supernatant was assayed for lipase activity. Also, absorbance of the culture was recorded at 600 nm to follow growth pattern.

3.1 Determination of protein

Protein estimation was done by the method of Lowry et al., using bovine serum albumin as the standard.

3.2 Purification procedure

Lipase was purified to homogeneity by ammonium salt precipitation and ion exchange column chromatography. Solid ammonium sulphate was added to the extract with stirring to bring the saturation to 60% and after standing it for overnight at 4°C, the precipitates that formed were collected by centrifugation. The precipitates were reconstituted in minimum amount of assay buffer and extensively dialyzed against 0.1 M Tris-HCl (pH 9.5) to remove ammonium sulphate. Finally, the lipase activity was assayed. A column (size 12 x 2 cm) was packed with DEAE-Cellulose (Sigma Chemicals Co., USA). The matrix was activated sequentially with 0.1 N NaOH and 0.1 N NaCl. Subsequently, column was equilibrated with 0.1 M Tris-HCl buffer (pH 9.5). The dialyzed sample (3 ml) was loaded onto the column. The column was eluted with 0.1 M Tris-HCl buffer (pH 9.5) containing NaCl gradient of 0.3, 0.5, 0.7 and 1 Molar. The eluent was collected in 3 ml fractions. All eluted fractions were assayed both for lipase activity as well as for their protein content. The fractions showing highest lipase activity were pooled. The specific activity of the purified enzyme was compared with that of initial crude enzyme and fold purification was calculated.

The molecular weight of the protein was determined by SDS-PAGE (12%) according to the method of Laemmli. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Molecular marker of range (29-97.4 kDa) was used as a marker.

4 Characterization of lipase

4.1 Effect of incubation time on lipase activity

To determine the optimum incubation time, the reaction
mixture was incubated for varying times i.e. 5, 10, 15, 20 and 25 min (each at an interval of 5 min) at 55°C in a water bath shaker and enzyme activity was measured under standard assay conditions.

4.2 Effect of pH and temperature
To evaluate the effect of reaction temperature and pH on lipase activity, the lipase was assayed at various temperature (45, 50, 55, 60, and 65°C, each at a difference of 5°C) and pH (7.5, 8.0, 8.5, 9.0, 9.5, and 10.0, each with a difference of 0.5 units) values.

4.3 Effect of metal ions on lipase activity
Each of the salts was separately added to the reaction mixture at a final concentration of 1.0 mM. After 10 min incubation at 55°C, the lipase activity was assayed.

4.4 Substrate specificity of lipase
To study the substrate specificity of the enzyme, different substrates p-nitrophenyl palmitate (p-NPP), p-nitrophenyl laurate (p-NPL), p-nitrophenyl benzoate (p-NPB) and p-nitrophenyl acetate (p-NPA), p-nitrophenyl formate (p-NPF) were used. The substrate concentration used was 20 mM and the enzyme activity was measured.

4.5 Determination of $K_m$ and $V_{max}$ of lipase enzyme from Bacillus aerius
The $K_m$ and $V_{max}$ of the enzyme was determined by measuring the reaction velocities at the different concentrations of the substrate i.e. 0.13, 0.27, 0.40, 0.54, 0.67, 0.81, 0.94, 1.08, 1.21 mM. The reciprocal of the reaction velocity was plotted against the reciprocal of the substrate concentration to determine the $K_m$ and $V_{max}$ values by Lineweaver-Burke plot.

4.6 Thermostability of lipase
To determine the thermostability of lipase, it was incubated at 50°C, 55°C and 60°C and enzyme activity was measured after every 30 min.

4.7 Effect of detergents and a protease inhibitor
To examine the effect of detergents, enzyme was pre-incubated in detergent (1%) such as Triton-X, Tween 20, Tween 80, SDS and a protease inhibitor, PMSF at 55°C for 1 h and enzyme activity was measured thereof.

5 Results and discussion
The supernatant of culture inoculated for 30 h with 7.5% (v/v) inoculum gave maximum lipase production (Fig. 1a, 1b). In a previous study, 24 h old inoculum of a Bacillus strain gave optimum lipase activity (Fig. 1a, 1b). In the present study, the lipase production after 6 h of incubation time was 0.164 IU/ml. Maximum lipase production was found to be after 48 h (0.695 IU/ml) at 55°C when production broth was inoculated with 7.5% (v/v) and 30 h old inoculum (Fig. 2a). When temperature and pH were optimized, maximum production of enzyme was observed at 55°C and pH 8.0 (Fig. 2b, 2c). Earlier, Lipase from Bacillus coagulans BTS-3 showed maximum activity at 55°C temperature and pH 7.5 (12). Good amount of lipase was produced in all the oils tested. Maximum lipolytic activity was obtained with cottonseed oil (1.0% v/v) suggesting its easy hydrolysis by Bacillus aerius (Fig. 3a). In a previous study, coconut oil at a concentration of 0.5% (v/v) was found to enhance the lipase production in the Bacillus strain B5 (13). However, the thermostable Bacillus sp. have been reported to produce
thermostable alkaline lipase with corn oil and olive oil as carbon sources. In the case of nitrogen source, yeast extract (0.5\% w/v) was found to enhance the enzyme production (Fig. 3b). This finding was in agreement with that of Al-Saleh and Zahran who showed that enzyme was produced in the absence of organic nitrogen by *Pseudomonas flourescens*\(^{21}\). In a similar study, Pimentel *et al.* recorded that a Brazilian strain of *Penicillium citrinum* produced a maximal lipase activity of 409 IU/mL in a medium that contained yeast extract (0.5\% w/v) as the nitrogen source and further decrease in yeast extract concentration reduced the attainable lipase activity also\(^{22}\).

5.1 Growth and enzyme production profile

The bacterial isolate showed a rapid increase in biomass during first 30 h of production, after which the growth

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**Fig. 2** (a) Effect of incubation time on the production of lipase from *Bacillus aerius* at 30°C and pH 7.0. (b) Effect of temperature on production of lipase from *Bacillus aerius* at pH 7.0 and incubation time of 48 h. (c) Effect of pH on production of lipase from *Bacillus aerius* at incubation time of 48 h and temperature 55°C. Inoculum age of 30 h and inoculum size of 7.5 \% v/v was used.

**Fig. 3** (a) Effect of different carbon sources (1\% v/v) on production of lipase from *Bacillus aerius*. Control lacks any carbon source. (b) Effect of different nitrogen sources (0.5\% w/v) on the production of lipase from *Bacillus aerius*. Control is devoid of nitrogen source. Temperature 55°C, pH 8.0, incubation time of 48 h was used.
became almost constant probably due to exhaustion of nutrients in the culture medium. Enzyme production started increasing after 24 h and reached to its maximum after a period of 30 h. Thereafter a decrease in activity was observed in the supernatant, which might be due to deactivation of enzyme or due to some toxic secondary metabolites released during growth. In a similar study earlier, a sharp decrease in lipase production was observed in case of *Bacillus licheniformis* B42 after 72 h which could be due to proteolytic degradation of enzyme system as well as exhaustion of nutrients in the medium.

### 5.2 Enzyme purification

Enzyme was purified by using DEAE-Cellulose column chromatography, in this, fractions between 7 to 18 showed maximum lipase activity and protein absorbance at 410 and 280 nm respectively (Fig. 4). The enzyme was purified to 9-fold with final lipase activity, protein content and specific activity of 85.44 IU, 7.92 mg and 10.85 IU/mg respectively (Table 1). Earlier, various lipases have been purified using ion exchange chromatography.

The purified lipase showed a single band in 12% SDS PAGE gel which suggested homogeneity of the purified enzyme. Its molecular weight was found to be 33 kDa (Fig. 5). Earlier, comparable molecular weights of lipase have been reported to be 32.2 kDa from *Aspergillus niger* NCIM 1207, 31 kDa from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3, 45 kDa from thermophilic *Bacillus sp.* J33, 66 KDa from *Bacillus sp.* THL027, 34 KDa from *Bacillus thermoleovorans* ID-1, and 30 KDa from *Acinetobacter calcoaceticus*. Other lipases that have been purified using single step of purification are lipase from *Bacillus pumilus* RK31 and from *Pseudomonas auruginosa* EF2.

### 6 Enzyme characterization

Lipases are highly diversified in their catalytic properties. Maximum lipase activity of *Bacillus aeuris* was obtained using 20 µl enzyme after 10 min of incubation at 55°C (Table 2). The lipase activity increased with increase in temperature from 45°C to 55°C but further increase in temperature adversely affected the lipase activity, it might be due to denaturation of enzyme at higher temperature. Lipase from *Bacillus licheniformis* B42 also showed optimal activity at 55°C and remained stable at a range of 50-90°C. The half life of enzyme was found to be 195, 112.5 and 97.5 min at 50, 55 and 60°C when incubated for 240 min respectively (Fig. 6). In earlier study, lipase from *Bacillus aeuris* was purified by DEAE-Cellulose column.

### Table 1 Summary of lipase purification from *Bacillus aeuris*.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total activity (IU)</th>
<th>Total Protein (mg)</th>
<th>Specific activity (IU/mg)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>1185</td>
<td>972</td>
<td>1.21</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>197.5</td>
<td>31</td>
<td>6.37</td>
<td>16.6</td>
<td>5</td>
</tr>
<tr>
<td>DEAE-Cellulose column chromatography</td>
<td>85.44</td>
<td>7.92</td>
<td>10.85</td>
<td>7.2</td>
<td>9</td>
</tr>
</tbody>
</table>

![Fig. 4](image.png) Elution profile of lipase from *B. aeuris* on DEAE-Cellulose column.

![Fig. 5](image.png) SDS-PAGE of purified lipase from *Bacillus aeuris*: Lane 1: Bangalore Genie Protein Marker (kDa). 97.4 kDa, Phosphorylase b; 66 kDa, Bovine Serum Albumin; 43 kDa, Ovalbumin; 29 kDa. Lane 2: dialyzed lipase. Lane 3: crude enzyme and Lane 4, 5: Purified lipase from DEAE-Cellulose Column.
Table 2  Effect of incubation time on activity of lipase from Bacillus aerius.

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Enzyme activity (IU/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.82 ± 0.005</td>
</tr>
<tr>
<td>10</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>15</td>
<td>0.63 ± 0.007***</td>
</tr>
<tr>
<td>20</td>
<td>0.61 ± 0.017*</td>
</tr>
<tr>
<td>25</td>
<td>0.48 ± 0.005***</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 3 replicates. *p < 0.05, **p < 0.01, ***p < 0.001 as compared to control.

Fig. 6  Thermostability of lipase from Bacillus aerius at 50°C, 55°C and 60°C.

Pseudomonas sp. was found to retain 86 and 52% of its maximum activity and remain stable for 4 h when it was incubated at 37 and 40°C. The activity of lipase is highly pH dependent, pH influences the structure and function of protein and hence governs its catalytic activity. The optimum pH for the lipase activity was found to be 9.5, and it retained 90% of its maximum activity at pH 10. Gilbert et al. reported that lipase from Pseudomonas aeruginosa EF2 had maximal activity at pH 8.5–9.0. The relative activity of enzyme from Bacillus aerius increased on exposure to most metal ions such as Cu²⁺ and Mg²⁺ and activity decreased with metal ions such as Na⁺, Ca²⁺ and Fe²⁺ (Table 3) while purified Bacillus aerius lipase showed a variable specificity towards various p-nitrophenyl esters (Table 4). Substrate specificity was controlled by molecular properties of the enzyme, structure of substrate and factors affecting the binding of the enzyme to the substrate. The high C-length (C: 16) ester (p-NPP) was more efficiently hydrolyzed than other esters. The enzyme was inhibited up to 70%, 59.5%, 79.5%, 85.3% and 89.0% in case of Triton X-100, Tween 20, Tween 80, PMSF (Protease inhibitor) and SDS respectively. SDS was found to be strong inhibitor as it resulted in change in local conformation in the active site by acting as competitive inhibitor of the enzyme possibly by partial reversible unfolding and subsequent inactivation. In another study, a thermostable lipolytic enzyme from a thermophilic Bacillus sp. showed maximum activity for p-nitrophenyl laurate. The Kₘ and Vₘₐₓ value of the enzyme was found to be 2.13 mM and 0.66 μmol/min/ml respectively. Earlier, the Kₘ and Vₘₐₓ value of lipase enzyme from Bacillus sp. was reported to be 2.5 mM and 0.4 μmol/min/ml respectively.

Table 3  Effect of different metal ions on activity of lipase from Bacillus aerius.

<table>
<thead>
<tr>
<th>Metal ion (1 mM)</th>
<th>Enzyme activity (IU/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.61 ± 0.065*</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>1.85 ± 0.015***</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.91 ± 0.01**</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.57 ± 0.005***</td>
</tr>
<tr>
<td>NaSO₄</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>Control</td>
<td>1.02 ± 0.02</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 3 replicates. *p < 0.05, **p < 0.01, ***p < 0.001 as compared to control.

Table 4  Effect of different substrates on lipase activity of Bacillus aerius.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl palmitate</td>
<td>100</td>
</tr>
<tr>
<td>p-nitrophenyl laurate</td>
<td>57.4</td>
</tr>
<tr>
<td>p-nitrophenyl benzoate</td>
<td>13.19</td>
</tr>
<tr>
<td>p-nitrophenyl acetate</td>
<td>12.05</td>
</tr>
<tr>
<td>p-nitrophenyl formate</td>
<td>7.13</td>
</tr>
</tbody>
</table>

Table 5  Effect of different detergents on lipase activity of Bacillus aerius.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Detergents</th>
<th>Enzyme Activity (U/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Triton X-100</td>
<td>0.57 ± 0.046**</td>
</tr>
<tr>
<td>2</td>
<td>Tween 20</td>
<td>0.77 ± 0.032**</td>
</tr>
<tr>
<td>3</td>
<td>Tween 80</td>
<td>0.39 ± 0.02***</td>
</tr>
<tr>
<td>4</td>
<td>PMSF</td>
<td>0.28 ± 0.005***</td>
</tr>
<tr>
<td>5</td>
<td>SDS</td>
<td>0.21 ± 0.02***</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>1.90 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of 3 replicates. ***p < 0.01, ****p < 0.001 as compared to control.
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7 Conclusion

The present study concluded that the Bacillus aerius isolate has shown the production of extracellular lipase when it was incubated at 55°C with pH 8.0 in nutrient agar medium. Further, enzyme was purified by DEAE-Cellulose column chromatography and has been found to be of M<sub>w</sub> 33 kDa in SDS-PAGE. As the Bacillus aerius was isolated from hot spring, lipase showed more activity as well as thermostability in comparison to many of the previously studied lipases till date and thus exhibited favorable kinetics to be used in industrial and environmental applications such as synthesis of medicinally important esters having anti-oxidant property, synthesis of biodiesel and flavored compounds.

Acknowledgments

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Conflicts of interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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