CHAPTER 4

Evaluation of lipase for its detergent additive capability
Chapter 4

ABSTRACT

Lipases are the enzyme of choice for many laundry detergent industries owing to their ability to remove triglycerides from the soiled fabric which reduces the usage of phosphate based chemical cleansers in the detergent formulation. A partially purified bacterial lipase from *Staphylococcus arletiae* JPBW-1 isolated from the rock salt mine has been assessed for the removal of triglyceride soil by developing a pre-soak formulation so as to use the enzyme as an additive in laundry detergent formulations. The effects of selected surfactants, commercial detergents and oxidizing agents on lipase stability were studied in a preliminary evaluation for use in detergent formulation. Partially purified lipase has been shown good stability in presence of surfactants, commercial detergents and oxidizing agents. Washing efficiency has been found to be enhanced while using lipase with 0.5 % non ionic detergent than the anionic detergent. The wash performance using 0.5 % Wheel with 40 H lipase at 40 °C within 45 min results in maximum oil removal (62 %) from the soiled cotton fabric. Hence, the present study opens the new era in enzyme based detergent sector for formulation of chemical free detergent using the alkaline bacterial lipase.

4.1 INTRODUCTION

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3.) are ubiquitous enzymes with industrial potential of synthesizing structural triglycerides, which are used as detergents and emulsifiers in nutrition and cosmetics (Andualem and Gessesse, 2012). Detergent enzymes constitute about 32 % of the total worldwide industrial enzyme production (Lomax et al., 1997). A major requirement for commercial lipases is thermal stability which would allow enzymatic reaction to be performed at higher temperature and would be helpful to increase conversion rates and substrate solubility. The importance of alkaline and thermostable lipases for different applications has been growing rapidly (Cherif et al., 2011). The usage of alkaline lipases in detergent formulations enhancing substantially which facilitates alternative for the phosphate builders in chemical detergent which are considered as major pollutants from detergent industry. Ideally, alkaline lipases in a detergent formulation should be stable over a broad range of
temperature, pH and compatible with surfactants, oxidizing agents at lower concentrations with broad substrate specificity (Jellouli et al., 2011). The detergent industries are relying on recombinant lipases (Lipex® and Lipolase® from Novozymes) for formulation of bio-detergents due to the suitability of these lipases for harsh conditions of detergent formulation ingredients such as surfactants, oxidizing agents (Saeki et al., 2007). Researchers are in search of enzymes from indigenous extremophile regions for better application in laundry detergent industry. Among different sources (fungal, yeast, bacterial), bacterial lipases received much attention for their substrate specificity and their ability to function in extreme environments of temperature, pH and surfactant and oxidizing agents tolerance (Horchani et al., 2012). Rathi et al., 2001 showed the application of bacterial lipase Burkholderia cepacia as an additive in detergent formulation which exhibits better stability towards commercial detergents and oxidizing agents in comparison to commercial Lipolase. In another study, Thirunavukarasu et al., 2008 have shown the use of Cryptococcus sp. S-2 lipase in detergent formulation and optimized washing conditions through response surface methodology. Moreover, bacterial lipases added to household detergents reduce or replaces synthetic detergents, which have considerable environmental problems (Basketter et al., 2012). Ideally, alkaline lipases are suitable candidates as a detergent additive for formulating a presoak formulation in detergent industry (Hasan et al., 2006). Hence, in the present study we are introducing an alkaline bacterial lipase produced by Staphylococcus arlettae JPBW-1. Lipase was further investigated in order to assess their compatibility with several other commercial surfactants, oxidizing agents and the well known commercial detergents and tested for its washing efficiency for removal of olive oil from soiled cotton fabric.

4.2 MATERIALS AND METHODS

4.2.1 Microorganism, chemicals and reagents

Staphylococcus arlettae JPRW-1 was used for the lipase production, which was isolated from the one and only rock salt mine of India, Darang, HP and deposited in MTCC, Chandigarh as Staphylococcus arlettae JPBW-1 MTCC 5589, maintained on Luria agar
slants at 4 °C. Olive oil used was the brand of Sos Cuetara, S.A. Figaro. Surfactants used were selected from the commercially available products; as nonionic surfactants: Tween 80 and commercial detergents of Indian market namely Ariel, Tide, Wheel active and Nirma (Procter and Gamble Home Products Ltd.), Rin Magic and Surf Excel (Hindustan Lever Ltd.), Sodium dodecyl sulphate (SDS) as anionic surfactant. p-nitrophenyl palmitate (p-NPP) was procured from Sigma, USA.

4.2.2. Lipase production and partial purification
Bacterial lipase was produced through submerged fermentation by cultivating 100 ml inoculum (48 h old) supplemented with 8 % soybean oil in a shaking flask (250 ml) with 100 ml of the LB broth medium. The culture was incubated for 3 h on a rotary shaker (125 rpm) at 37 °C. After 3 h, the fermented broth was centrifuged at 5367 g for 15 min at 4 °C and the cell-free supernatant was used for estimation of lipase activity and partial purification (Ammonium sulphate precipitation, 60 %). The lipase has pH optima of 11.0 and activity in a broad temperature range of 25-90 °C (Chauhan and Garlapati, 2013).

4.2.3 Lipase assay
Lipase activity was determined using p-NPP as substrate (Garlapati and Banerjee, 2010). One unit (U) of lipase activity was expressed as the amount of enzyme that liberates one micromole of p-nitrophenol released per minute under the assay conditions.

4.2.4 Compatibility of lipase with surfactants and commercial detergents
To investigate the compatibility of lipase in various surfactants and commercial detergents, respective surfactants and detergents were added to the reaction mixture at a concentration of 7 mg/ml and assayed under standard assay conditions and expressed as percent relative activity. The endogenous lipases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65 °C prior to the addition of the enzyme preparation. To determine the stability, an aliquot of enzyme sample (50 U/ml) was incubated with equal volumes of detergent solution (7 mg/ml of respective detergent) in Tris- HCl buffer (0.1 M, pH 8.0) for 1 h at 30 °C. The relative activity (%)
of each sample was determined and compared with the control without detergent. The relative activity of control was defined as the enzyme activity without detergent, incubated under the similar conditions and was taken as 100%.

4.2.5 Compatibility of lipase with oxidizing agents

Lipase (50 U/ml) compatibility in presence of oxidizing agents was determined in Tris-HCl buffer (0.1M, pH 8.0) containing 0.5–2.0% (v/v or w/v) of hydrogen peroxide, sodium perborate and sodium hypochlorite for 1 h at 30°C and relative activity was estimated and compared with the control without oxidizing agent. The relative activity of control was defined as the enzyme activity without oxidizing agent, incubated under the similar conditions and was taken as 100%.

4.2.6 Preparation of olive oil soiled cotton fabric

The cotton fabric (5cm X 10cm) to be soiled was highly defatted in boiling chloroform for four hr. This treatment was repeated three times. The cotton fabric was soiled by spotting with 0.5 ml of olive oil benzene solution (100 mg/ml conc.) with micropipette two times.

4.2.7 Preparation of washing solution

Four kinds of washing solution were prepared their compositions are shown in Table 4.1 for making 100 ml of respective washing solution. Solution B-D-L, which contained the buffer solution, the surfactants solution and the lipase solution, was prepared in the following manner. The buffer solution and the surfactant solution were measured into an Erlenmeyer flask with ground stopper and preheated at 37°C for 10 min, followed by the addition of the lipase solution. Solutions B-L, B-D and B were prepared in the same manner. The volume of the final solutions was adjusted to 100 ml by adding distilled water. Then, 10 pieces of the soiled fabric was put into flask. For selecting the best process condition (conc. of detergent, activity of lipase, washing temperature and washing time) initially washing compositions of Table 4.1 was used. After selection of one best process condition through changing one variable at a time approach, the best condition was used for the selection of next process condition by utilizing the proportion of Table 4.1 for making the detergent solutions.
Table 4.1: Washing solutions and its composition for making 100 ml of washing solution

<table>
<thead>
<tr>
<th>Components</th>
<th>B^a</th>
<th>B^a+ L^b</th>
<th>B^a+D^c</th>
<th>B^a+ D^c+ L^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (0.1M, pH 8.0)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Detergent (0.5%)</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Lipase (500U/ml)</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water</td>
<td>60</td>
<td>30</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

# ^aBuffer ^bLipase ^cDetergent

4.2.8 Washing procedure and olive oil determination

The soiled fabric was washed at 37 °C for 20 min by incubator with the shaking of 100 rpm. The fabric was rinsed thrice each rinsing was with 100 ml of distilled water at 37 °C for 2 min. Then, fabric was air dried. Washing also was done at different temperature and time interval, as well as by using the lipase solution and the detergent solution, both in different concentrations. Olive oil was extracted from the fabric with petroleum ether for six hours in Soxhlet extractor. After petroleum ether was completely evaporated from the extract and weight of olive oil was determined.

Calculation of removal: The removal of olive oil was calculated by equation, based on the weight of the total fatty acids on the fabric before and after washing.

\[
\text{Removal (\%) = } \left( \frac{W_i - W_r}{W_r} \right) \times 100
\]

Where \( W_i \) = weight of total olive oil before washing (mg) and \( W_r \) = weight of total olive oil after washing (mg).

4.3 RESULTS AND DISCUSSION

4.3.1 Compatibility of lipase with surfactants and commercial detergents

For effective use under harsh detergent industry conditions, lipolytic enzyme must be compatible and stable with all commonly used detergent compounds such as surfactants which mainly present in any detergent formulation (Kamini et al., 2000). The \textit{S. ariellicae} lipase was tested for its potential as an additive in detergents. The enzyme showed excellent compatibility and stability in the presence of ionic and nonionic surfactants as well as in commercial detergents (Fig.4.1). The enzyme showed increased stability in
presence of SDS, Tween 80 and similar results were reported for lipases from *Aspergillus sp.* and *Rhizopus sp.* (Saisubramanian et al., 2006 and Derevenda et al., 1994). Among different detergents, wheel showed a maximum % increase in activity, while SDS exhibited an increase of approximately 6 % in activity over control (Fig.4.1). However, the lipase activity of *Ralstonia picketti* (Hemachander and Puvanakrishnan, 2000) and *Aspergillus carneus* were inhibited in the presence of SDS (Saxena et al., 2003) while the activity was increased in *H. lanuginosa* (Omar et al., 1987).

![Graph](image)

**Figure 4.1:** Compatibility of *S. arletiae* lipase with surfactants and detergents. For the control, lipase was incubated with buffer devoid of surfactants and detergents and its activity was taken as 100 %. All values are represented as mean ± s.d of three replications.

### 4.3.2 Lipase compatibility with oxidizing agents

Bleach stability of the enzyme was also checked in the presence of hydrogen peroxide, sodium hypochlorite, sodium perborate and sodium peroxide. The lipase was highly stable towards oxidizing agents at 1.5 % concentration for 1 h at 30 °C and it retained 92 % of activity even at 2.0 % concentration of hydrogen peroxide, while activity was gradually decreased with increase in concentrations of sodium perborate and sodium hypochlorite from 1.0 to 2.0 % (Fig.4.2). Remarkably, the present lipase exhibited better resistance towards strong oxidizing agents especially hypochlorite (95 % activity at 1.0 % concentration) compared to the relative activity of lipolase (Novozymes, Denmark), which exhibited 43 % activity after 1 h treatment as reported by Rathi et al.,...
2001. Therefore, the stability of oxidations is an important characteristic required for an enzyme to be incorporated into a detergent.

![Graph showing the effect of oxidizing agents on lipase activity.](image)

Figure 4.2: Compatibility of *S. arlettae* lipase with oxidizing agents. For the control, lipase was incubated with buffer alone without oxidizing agent and its activity was taken as 100%. All values are represented as mean ± S.D of three replications.

### 4.3.3 Effect of detergent and its concentration on oil removal

Effect of different commercial detergents on oil removal has been shown in Table 4.2. Among all detergents (0.3%), wheel exhibited highest oil removal (52%) from soiled cotton fabric and it has been chosen for subsequent studies. In detail, the lipase was more efficient with the nonionics than with the anionics. This is because the activity of the lipase is less inhibited by the nonionics than by the anionics (Sajna et al., 2012 and Flipsen et al., 1998). The relation between the concentration of surfactant in the presence of the lipase and the removal of olive oil is shown on Fig.3. At any concentration of any wheel detergent, the removal of olive oil with solution B-D-L was always higher than the solution B-D. Thus, it was proven that the lipase was effective with any detergent system at any concentration.
Table 4.2: Effect of lipase on removal of olive oil from cotton fabric with various detergents

| Detergent  | Oil removal (%) |  |  
|------------|-----------------|---|---|
|            | B^a + D^b       | B^a + D^b + L^c |   |
| Tide       | 39.0            | 50.2 |   |
| Run magic  | 33.2            | 45.5 |   |
| Surf excel | 36.4            | 47.5 |   |
| Ariel      | 31.2            | 43.0 |   |
| SDS        | 30.6            | 39.5 |   |
| Tween 80   | 28.3            | 38.0 |   |
| Wheel active | 41.5      | 52.0 |   |
| Nimia      | 30.2            | 41.2 |   |

# ^aBuffer; ^bDetergent; ^cLipase

It is proven, based on results of this study, at 0.5 % concentration of wheel that the lipase from *Staphylococcus arlettae* improves the removal of olive oil from cotton fabric by 37 (B + D) to 49 % (B + D + L) under the conditions of 30 units as the lipase concentration, 37 °C as washing temperature and 30 min of longer as washing time (Fig. 4.3). It can be expected that the lipase will be used for laundry detergents.

![Graph showing the effect of detergent and its concentration on oil removal](image)

**Figure 4.3:** Effect of detergent and its concentration on oil removal (%) (Experimental conditions: Lipase amount 30 U; Washing temperature 37 °C; Washing time 30 min). All values are represented as mean ± s.d of three replications

**4.3.4 Effect of lipase amount on oil removal**

The relation between the lipase concentration and olive oil removal from stained fabric has been depicted in Fig. 4.4. In both cases, oil removal increases with lipase concentration till attaining the equilibrium state at a concentration of more than 40 units. The equilibrium attainment after certain lipase concentration has been depends on
the initial rate of hydrolysis of triglyceride by lipase based on the interface area between insoluble triglyceride and the aqueous solution of lipase. The surface area of a given amount of olive oil will be constant after a certain concentration of lipase with which interface is saturated. As shown in Fig.4, the addition of the lipase brought an improvement from 26 % to 55 % without the detergent and with detergent. Enhanced results of lipase in combination with a commercial detergent has been reported in several instances such as usage of *Pseudozyma* sp. NII 08165 (Sajna et al., 2012) produced biosurfactants and *Pseudomonas aeruginosa* lipases (Grbavcic et al., 2011).

![Figure 4.4: Effect of lipase amount on oil removal (Experimental conditions: Detergent concentration 0.5 %; Washing temperature 37 °C; Washing time 30 min). All values are represented as mean ± s.d of three replications.](image)

**4.3.5 Effect of washing temperature on oil removal**

The results of oil removal with and without lipase at different temperatures have been shown in Fig 4.5. A maximum oil removal in case of B-L as a washing solution has been noticed at 37 °C. In the case of B-D washing solution, it has been seen that better oil removal has been takes place at higher washing temperature only (Horčani et al., 2009 and Romdhane et al., 2010). With solution B-D-L containing the wheel and the lipase, the removal was higher at any washing temperature than with any other solution. However, the contribution of the lipase to the removal of olive oil has been seen most significantly at the optimum temperature of the lipase of 40 °C.
Figure 4.5: Effect of washing temperature on oil removal (Experimental conditions: Detergent concentration 0.5 %; Lipase amount 40 U; Washing time 30 min). All values are represented as mean ± s.d of three replications.

4.3.6 Effect of washing time on oil removal

The effect of washing time on oil removal has been depicted in Fig. 4.6. It has been observed that, on longer washing time, only B-L solution worked properly for oil removal from stained cloth. On the other hand, the removal with solution B-D became nearly constant after 20 min. Finally, it has been observed that utilization of B-D-L solution results with enhanced oil removal (62 %) in 45 min of washing cycle. Thus, the significant contribution of lipase in oil removal has also been observed with prolonged washing time. Sajna et al., 2012 has been reported that the enhanced stain removal was seen with increased wash time using Pseudozyma sp. lipase as a detergent additive. Similar enhancement with increasing wash time has also been reported by Grbavčić et al., 2011 in case of Pseudomonas aeruginosa lipase biodetergent study.
Figure 4.6: Effect of washing time on oil removal (Experimental conditions: Detergent concentration 0.5 %; Lipase amount 40 U; Washing temperature 40 °C). All values are represented as mean ± s.d of three replications.

4.4 CONCLUSIONS

Lipase from *S. arleltiae* JPBW-1 was an ideal candidate for use in laundry detergent formulations, since it possessed better stability with surfactants, commercial detergents, bleaching and oxidizing agents. It has been found that the lipase was more compatible with nonionic surfactant than the anionic counterpart, which makes it a novel lipase for further commercial utilization in detergent formulations. The results of this study show that the lipase from *S. arleltiae* improves the removal of oil from cotton fabric by 21 % as an additive commercial detergent viz. 0.5 % Wheel under the optimum conditions of 40 U of lipase in Tris-HCl buffer (0.1M, pH 8.0) at 40 °C as washing temperature in a washing time of 45 min. Hence, lipase from *S. arleltiae* has been the ideal choice for formulating an environmentally friendly detergent formulation with the objective of oil removing from soiled fabrics.