Chapter 9

Characterization of alkaline lipases from two isolates
Lipases possess a wide range of catalytic properties which are mostly strain-dependent. They have frequently been used in the form of a crude extract for synthesis of chiral building blocks and enantiomeric compounds. Catalytical properties such as specificity, enantioselectivity and operational parameters like thermostability and optimum pH, among others, are relevant because they define the enzyme application range and type of process (Pera et al., 2006).

Although a large number of bacterial lipases with many different enzymological properties have been produced (Jaeger and Reetz, 1998), very few lipases with optimum activity under alkaline conditions were reported so far (Winkler and Stuckmann, 1979). The knowledge and expertise in new microorganisms capable of producing lipases as well as a greater understanding of their properties would be very useful in the application of such systems for the purpose of finding use in industries.

In this Chapter, alkaline lipases from the two *Thraustochytrium* spp. AH-2 and TZ have been characterized. Their properties such as optimum assay temperature, pH, basic enzyme kinetics and biotechnologically significant characteristics (pH and temperature stability metal tolerance and substrate specificity) are detailed. Based on the results, the more active of the two lipases would be selected for further studies.

9.1 Materials and Methods

Crude enzyme extracts from the two isolates TZ and AH-2 were produced as described in Chapter 7. All experiments were carried out in duplicate and each
data set is representative of two to three independent experiments. Each parameter is reported as the mean ± S.D.

9.1.1 Effect of enzyme concentration and time

Different amounts of enzyme protein were assayed in borate buffer in a final volume of 2.5 ml under the standard conditions as described in Section 7.1.3. The enzyme preparation was assayed for time periods up to 40 min in order to select the most appropriate assay time.

9.1.2 Effect of temperature on enzyme activity and stability

The experiment was carried out by incubating ideal amounts of the crude enzyme protein at different temperatures (28°C-90°C) for 10 min with p-NPP in the presence of borate buffer (50 mM, pH 9.0) for assaying the lipolytic activity. Thermostability was analyzed after subjecting the culture filtrate to heat treatment for 10 min at various temperatures ranging from 40 to 90°C. The precipitated protein was then centrifuged down and the residual activity of the supernatant assayed under the standard conditions. The time course of thermostability was studied by subjecting the crude enzyme to heat treatment for different time periods at a selected temperature, prior to assaying under the standard conditions.

9.1.3 Effect of pH on enzyme activity and stability

The enzyme reaction was carried out in various buffers viz, acetate buffer (50 mM, pH 3.5-5.0), phosphate buffer (100 mM, pH 6.0-8.0) or borate buffer (15 mM, pH 8.0-10.0), other assay conditions being maintained as above.
The pH stability of the enzyme was analyzed by incubating the preparation with an equal volume of the respective buffer (pH range 3.5-10) for 1 h at room temperature (30 ± 2°C). The residual activity was then measured as per the routine assay procedure.

9.1.4 Substrate concentration studies

The enzyme extract was incubated with different concentrations of p-NPP during the assay under the above standard conditions. The kinetics of the reaction were studied by subjecting the data to Michaelis-Menten analysis.

9.1.5 Effect of EDTA and metal ions

The enzyme preparation from the isolate TZ was pre-incubated with varying concentrations of EDTA or metal ions (30 min, 30°C) and the residual activity of the enzyme were then assayed.

9.2 Results and Discussion

Each application of the enzyme requires unique properties with respect to activity, stability, temperature and pH dependence. The following properties of the lipases from the selected isolates AH-2 and TZ were thus studied with a view to characterize them and to understand their potential for industrial applications.

9.2.1 Effect of enzyme concentration and time

When different amounts of enzyme protein were assayed under the standard conditions as described above, the activity was found to be linear up to about
0.04 mg protein for lipases from both the isolates TZ and AH-2 (Fig 9.1a, b). All further assays were carried out using protein concentrations in this linear range. This dependence of the measured release of p-nitrophenol on enzyme protein concentration also confirmed that the estimation was an enzymatic process and ruled out any artifacts of measurement procedures.

This displacement from the linear curve as seen in Figs 9.1a and b could be due to i) the presence of small amounts of some impurity in one of the components of the incubation mixture other than the enzyme solution itself. This would poison the first amounts of added enzyme and it is only when enough of the enzyme is added to combine with the whole of the impurity that further amounts of enzyme will remain active or ii) the presence of a dissociable activator or coenzyme in the enzyme preparation (Dixon & Webb, 1979).

The time course of lipase activity (Fig 9.2) showed that the activity from both isolates tended to plateau off beyond 10 min. An assay time of 10 min was hence chosen for all further experiments.

9.2.2 Effect of temperature
The effect of assay temperature on enzyme activity was studied by carrying out the assay at different temperatures under the standard experimental conditions. From Fig 9.3, it is seen that the enzyme was maximally active at 50°C beyond which the activity started declining. This temperature of 50°C was thus chosen as the standard assay temperature for the lipases from both the *Thraustochytrium* spp. being studied.
Fig 9.1a Enzyme concentration curve for lipase from isolate TZ

Fig 9.1b Enzyme concentration curve for lipase from isolate AH-2

Lipase assay carried out at 50°C, 10 min, pH 9.0 using p-NPP as substrate and varying concentration of enzyme protein from isolates TZ and AH-2.
Fig. 9.2 Time course of enzyme activity

Assay conditions — pH 9.0, 2-40 min, temperature range 50°C using p-NPP as substrate and varying assay time as indicated.

Fig. 9.3 Effect of temperature on enzyme activity

Lipase assay was carried out at pH 9.0 for 10 min at varying temperatures
The lipases produced by *Fusarium oxysporum* f. sp. *line* and *F. oxysporum* f. sp. *vasinfectum* showed optimum activity at 42°C and 45°C, respectively (Hoshino, 1992; Rapp, 1995). Toida *et al.* (1998) found the maximum lipase activity of *A. oryzae* at 40°C and at higher temperatures, particularly above 55°C, a marked fall in enzyme activity was observed. The lipase from *P. simplicissimum* lost its activity completely at 60°C (Sztajer *et al.*, 1992). On the contrary, lipases from our *Thraustochytrium* sp. retained 71% (for isolate TZ) or 35% (for isolate AH-2) activity at 55°C. Even at 60°C the lipase from isolate TZ was 42% active.

According to Razak *et al.*, (1997) very few fungal lipases exhibit temperature optima above 40°C. Generally lipases from *A. niger* strains have been reported to be active between 40 and 55°C (Kamini *et al.*, 1998). Saxena *et al.* (2003) reported that *Aspergillus carneus* lipase showed optimum activity at 37°C. The lipase activity from *Rhizopus* sp. had a 50°C temperature optimum (Maria and Gláucia, 2006).

The optimal reaction temperature for the lipases from *Thraustochytrium* spp. reported in this study is also higher than that reported for many bacterial lipases (Snellman *et al.*, 2002) under similar experimental conditions. Activity at high temperature is a useful characteristic for lipases that are used in detergent formulations.

Thermal stability results (Fig 9.4a) showed that the lipase activity was maximally stable for at least 10 min up to 50°C or at 45°C for the enzymes from
Fig. 9.4a Thermal stability

Residual activities of enzymes from the isolates AH-2 and TZ after heat treatment for 10 min at 45°C and 50°C respectively. The activity of untreated enzyme was taken as 100%.

Fig. 9.4 b Time course of thermal stability

Residual activities of enzymes from the isolates AH-2 and TZ after heat treatment at 45°C and 50°C respectively for various time period. The activity of untreated enzyme was taken as 100%.
the isolates TZ and AH-2, respectively. When heat treatment at these respective temperatures was given for different time periods, it was observed (Fig 9.4b) that the enzymes from the isolates TZ and AH-2 could retain about 95 and 75% activity respectively, following a 20 min heat treatment.

The comparatively better thermotolerance during the assay (Fig.9.3) especially for the lipase from isolate AH-2, attributed to partial protection afforded by the presence of the substrate.

In recent years there has been a great demand for thermostable enzymes in industrial fields. Thermal stability of an enzyme is obviously related to its structure and is also influenced by environmental factors such as pH and the presence of metal ions. At least in some cases, thermal denaturation appears to occur through intermediate states of unfolding of the polypeptide. Attempts are being made to protein engineer lipases for improved thermal stability (Zhu et al., 2001). Thus the observed thermotolerance of the extracellular lipase from *Thraustochytrium* sp. may be of significance.

### 9.2.3 Effect of pH on enzyme activity and stability

The lipolytic activity from isolates TZ and AH-2 was assayed at pH values ranging from 3.5-10 as described earlier and the results are presented in Fig 9.5a. The optimum pH value was found to be 9.0 for the enzymes from both the isolates TZ and AH-2 and more than 50% of the maximum activity was retained even at pH 11. Stability experiments showed that both the enzymes were highly stable at alkaline pH range, retaining 70 and 92% activity of the
enzymes, from the isolates AH-2 and TZ, respectively, after 1 h incubation (Fig 9.5b). These results reconfirm the alkaline nature of the lipases under study.

Most of the extracellular lipases have been known to possess neutral or alkaline pH optima (Sugiura et al.,1977; Yamamoto and Fujiwara,1988; Shabtai and Daya-Mishne,1992; Lesuisse et al.,1993; Schuepp et al.,1997; Lee et al.,1999). The optimal pH for the crude lipase activity were 7.0 for Pe wortmanii (Costa and Peralta,1999), 8-9 for P. putida 3SK (Lee and Rhee 1993), 6.5 for P. burtonii (Sugihara et al. 1995), 9-10 for B. stearothermophilus (Kim et al.,1998), 5.5 for A. oryzae (Toida et al., 1998), 7.0 for M. hiemalis (Hiol et al.,1999) and 5-6 for A. niger (Namboodiri and Chattopadhyaya, 2000).

The enzyme from the fungus Cunninghamella verticillata was stable up to pH 9.0 and lost its activity when the pH was raised above 9 (Gopinath et al., 2002). Extracellular lipase of F. oxysporum f. sp. vasinfectum showed stability over the pH range of 4 to 10 during 1h incubation at 30°C (Rapp, 1995). On the other hand, others strains of F. oxysporum showed stability only at alkaline pH (Hoshino et al, 1992).

Triglycerides present in stains on fabrics are difficult to remove because they are hardly saponified compared to fatty acids. Lipases functioning would be useful in this regard. The pH studies on the lipase from the Thraustochytrium spp. AH-2 and TZ revealed that these enzymes could work best in alkaline environments and this characteristic of the enzyme would therefore have many
Fig. 9.5a Effect of pH on enzyme activity

Lipase assay carried out at 50°C and 10 min at varying pH

Fig. 9.5b Effect of pH stability on enzyme activity

The crude enzyme extracts were incubated in buffers of pH 3.5-10 for 1 h at room temperature and the residual activity was calculated. The activity of un incubated enzyme was taken as 100%.
applications in industry and also in reactions performed under alkaline conditions.

A lipase that is stable at high alkaline conditions and high temperature is however rare (Savitha et al., 2007) while in the present study we have isolated *Thraustochytrium* spp. which produced an inducible, extracellular, alkalophilic and thermostolerant lipase. There have been very few reports available to date with molds having alkalophilic and thermostable lipase. The lipases from the *Thraustochytrium* sp. reported in this research work could therefore be exploited for commercialization as enzymes of these characteristics find immense application as additives in washing powders.

### 9.2.4 Substrate concentration studies

Varying concentrations of *p*-NPP were used for the assay and the kinetics of the reaction were studied. The enzymes from both *Thraustochytrium* spp. showed saturation kinetics and the $K_m$ values were 0.182 and 0.5 $\mu$mol/ml for the enzymes from the isolates TZ and AH-2, respectively (Fig 9.6a, b).

The $K_m$ of lipase from *Fusarium solani* was 1.8 mM (Maia et al., 2001), from *Bacillus coagulans* MTCC-6375 was 28 mM (Kanwar et al., 2006) and from *Acinetobacter* sp.B2 was 21.8 mM (Korean medical database, 2004).
Fig. 9.6a Lineweaver-Burk plot for the lipase from isolate TZ

Fig. 9.6b Lineweaver-Burk plot for the lipase from isolate AH-2
9.2.5 Effect of EDTA and metal ions

The effect of EDTA on lipase activity as shown in Fig.9.7 revealed that even at the lowest concentration (0.25mM) of EDTA tested, 50% of the activity was lost and as the concentration increases complete loss of activity was observed. Inhibition by EDTA probably results from its access to Ca\(^{2+}\) or Mg\(^{2+}\) binding site and ion removal. A similar inhibition by EDTA has been reported for a few other lipases also (van Oort et al., 1989, Baral and Fox, 1997, Sharon et al., 1998). An extracellular lipase, LipA, of Acinetobacter sp. RAG-1 was totally inactivated at 1mM EDTA. (Snellman et al., 2002).

The effect of metal ions on lipase activity is shown in Table 4.1. Added magnesium ions marginally stimulated the enzyme activity. With calcium ions also, the activity was stable although no stimulation of activity was observed. There was total inhibition of activity by mercury, zinc, manganese, ferric and copper ions even at low concentrations of 1 mM.

Cofactors are generally not required for lipase activity, but divalent cations such as calcium and magnesium are known to often stimulate enzyme activity in microorganisms. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids. Rathi et al. (2001) observed stimulation in lipase production from Burkholderia sp. in the presence of Ca\(^{2+}\) and Mg\(^{2+}\). Sharma et al. (2002) also reported stimulation of lipase production from Bacillus sp. RSJ1 in the presence of calcium chloride. Calcium-stimulated lipases have been reported in the case of B. subtilis 168 (Lesuisse et al., 1993), B. thermoleovorans ID- 1 (Lee et al., 1999), S. hyicus (van Oort et
Figure 9.7 Effect of EDTA on enzyme activity from the isolate TZ

The crude enzyme extract from the isolate TZ was incubated in various concentrations of EDTA for 30 min at 30°C and the residual activity was assayed. The activity of untreated enzyme was taken as 100%.

Table 9.1 Effect of metal ions

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn$^{2+}$</td>
<td>1</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>1</td>
<td>113.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100.4</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>92.4</td>
</tr>
</tbody>
</table>
al., 1989) and Acinetobacter sp. RAG-1 (Snellman et al., 2002). In contrast, the lipase from *P. aeruginosa* 10145 (Finkelstein et al., 1970) was inhibited by the presence of calcium ions.

Further, lipase activity is in general drastically inhibited by heavy metals (Kanwar et al. 2002) and metal ions like Zn\(^{2+}\) and Cu\(^{2+}\) are reported by several workers to have slight inhibitory effect on *Pseudomonas* lipases (Yamamato and Fujiwara, 1988, Lizumi et al. 1990, Kumura et al. 1993, Chartrain et al. 1993). The present study also yielded similar results. Metal ions tested may have variable affects on lipase aggregation and on the substrate–water interface through interaction with free fatty acids (Snellman et al., 2002).

The characteristic features of the two alkaline lipases studied are summarized in Table 9.1.
Table 9.2 Comparison of characteristics of crude enzymes from the isolates AH-2 & TZ

<table>
<thead>
<tr>
<th>Property</th>
<th>Enzyme from AH-2 isolate</th>
<th>Enzyme from TZ isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction time</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Optimum temp</td>
<td>50°C</td>
<td>50°C</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>$K_m$ (μ mol/ml)</td>
<td>0.5</td>
<td>0.182</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>at 45°C for 15 min</td>
<td>at 50°C for 15 min</td>
</tr>
<tr>
<td>pH stability</td>
<td>Stable at alkaline pH</td>
<td>Stable at alkaline pH</td>
</tr>
<tr>
<td></td>
<td>(≥70%)</td>
<td>(≥90%)</td>
</tr>
</tbody>
</table>

Between the two enzymes, the superior one (the lipase from isolate TZ) was selected for further studies in the following Chapters.