Chapter 8

Optimization of growth conditions favoring maximum lipase production by selected isolates
Microbial lipases are mostly produced in submerged culture (Ito et al., 2001) but solid state fermentation methods (Chisti, 1999) could also be used. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production in submerged cultures. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration (Elibol and Ozer, 2001). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield although a few authors have reported good yields in the absence of fats and oils. Most microbial lipases are extracellular. Optimisation of fermentation conditions for microbial lipases is of great importance, since culture conditions influence the properties of the enzyme produced. The amount of lipase produced is dependent on several environmental factors such as cultivation temperature, pH, nitrogen composition, carbon and lipid sources, concentration of inorganic salts and the availability of oxygen.

A few researchers have performed systematic medium optimization and fermentation studies for lipase production. The organisms are normally grown in a complex nutrient medium containing a carbon source (usually oil), a nitrogen source (organic/inorganic), phosphorus source (sodium or potassium phosphate) and mineral salts, supplemented with micronutrients (MgSO4 or CaCl2). The pH of the medium is generally maintained around 7.0. A pH range between 8.0-10.0 has been used for lipase production by alkalophillic bacteria (Horikoshi, 1990).
Optimization of growth conditions of the two selected thraustochytrid isolates with respect to physico-chemical parameters such as harvesting time, pH, shake vs static conditions, temperature and salt concentration of the culture medium as well as varying compositions of the medium is reported in this Chapter.

8.1 Materials and Methods

All chemicals used were of analytical grade and glass double distilled water was used at all times. In all experiments, the measurements were carried out with duplicated parallel cultures. Each data point plotted is a representation of mean ± S.D. of values analyzed in replicate from two independent experiments.

8.1.1 Harvesting Time

MV Broth containing 0 -1 % olive oil was inoculated with isolates TZ or AH-2, kept on a rotary shaker at room temperature and the enzyme production was monitored every 24 hr for a seven day period.

8.1.2 Growth pH

Production medium with pre-adjusted pH (3-11) with the respective buffers was inoculated with each of the isolates and kept under agitation for 4 days at room temperature. The enzyme production was then determined as described in Chapter 7.

8.1.3 Effect of temperature

MV Broth with 0.5% olive oil was inoculated with isolates TZ and AH-2, kept under the above mentioned optimized conditions at different temperatures and the enzyme production was determined.
8.1.4 Effect of crude salt concentration

MV Broth at various crude salt concentrations and containing 0.5% olive oil was used to grow the isolates under optimized conditions as above and the enzyme production was determined.

8.1.5 Effect of inducers

The enzyme production at 0.5% concentration of various oils (groundnut, sunflower, coconut, palm, gingely and olive oil) provided as inducers was studied under the above optimized culture conditions.

8.1.6 Medium composition

Different composition of media used for the enzyme production under the above optimized conditions were:

1. Crude sea salt (3.4%) + Glucose (0.4%) + 0.5% olive oil
2. Crude sea salt (3.4%) + Glucose (0.4%) + 1.0% olive oil
3. Crude sea salt (3.4%) + Peptone (0.15%) + 0.5% olive oil
4. Crude sea salt (3.4%) + Peptone (0.15%) + 1.0% olive oil
5. Crude sea salt (3.4%) + Yeast extract (0.01%) + 0.5% olive oil
6. Crude sea salt (3.4%) + Yeast extract (0.01%) + 1.0% olive oil
7. MV medium + 0.5% olive oil
8. Crude sea salt (3.4%) + Peptone (0.15%) + Yeast extract (0.01%) + 0.5% olive oil

8.2 Results and Discussion

In the present study, the various physico-chemical parameters optimized to obtain maximum alkaline protease production by the two thraustochytrids were
a) harvesting time  b) growth pH  c) agitation  d) temperature  e) concentration of crude salt  f) effect of inducers  and  g) composition of the medium

8.2.1 Time course of enzyme production

Lipase production was studied for different time periods at room temperature in MV medium containing 0, 0.25, 0.5 and 1.0 % olive oil. The results showed that with 0.5% olive oil as inducer, the enzyme production by the isolates TZ (Fig 8.1) and AH-2 (Fig 8.2) was maximum at an optimum period of 96 h. There was no production of the enzyme in the absence of olive oil (data not shown) indicating the inducible nature of the enzyme production by both the isolates. The enzyme production which was initiated at as early as 24 h of incubation gradually reached a maximum by 96 h. So for all further experiments, time period of 96 h was chosen for maximum enzyme production by both isolates.

Similar results were reported for the extracellular lipase from *Fusarium solani* FS1 cultures where maximum activity was elicited after a 96 h incubation in medium containing 3% (w/v) peptone supplemented with different carbon sources (Maia et al., 1999). In *Penicillium wortmanii* cultures, maximum lipase production was obtained in a 7-day old culture using olive oil (0.5%) as the carbon source (Costa and Peralta, 1999).

The inhibition of the synthesis of lipases at higher olive oil concentrations could be due to poorer oxygen transfer into the medium. Low oxygen supplies can alter fungal metabolism and consequently, the production of lipases (Lima et al., 2003). A decline in enzyme production was observed after four days by both
Fig. 8.1 Time course for the production of alkaline lipase by the isolate AH-2

Fig. 8.2 Time course for the production of alkaline lipase by the isolate TZ

Production conditions - The isolates were grown in MV medium supplemented with varying concentrations of olive oil for 7 days and the lipase activity was determined at regular intervals of 24 h.
Thraustochytrium spp. in the present study which could be attributed to end product repression by the accumulation of fatty acids. A similar kind of repression was reported in Pseudomonas aeruginosa EF2 (Gilbert et al., 1991), P. fragi (Smith and Alfrod, 1966) and Rhizopus japonicus (Aisaka and Terada, 1979). Higher concentrations of olive oil also suppressed lipolytic activity in Penicillium aurantiogriseum (Lima et al., 2003).

8.2.2 Optimum pH of the production medium

When the pH of the production medium was pre-adjusted to values ranging from 3.5-10, maximum lipase production was observed at pH 6.0 (Fig 8.3). On either side of this pH, the activity elicited dropped sharply by almost 50%. For all further experiments, a pH of 6.0 was maintained for the production medium.

The initial pH of the growth medium is important for lipase production. Maximum activity was observed at pH values beyond 7.0 for P. fragi and at pH 9.0 for P. aeruginosa wherein development of acidity in media reduced lipase activity (Saxena et al., 1999). In contrast, maximum growth was reported for Staphylococcus lipolytica, Mucor caseolyticus, M. racemosus, M. hiemalis Bacidiobolus licheniformis, Aspergillus wentii, Rhizopus nigricans, R. oligosporus, and Pseudomonas aeruginosa EF2 at acidic pH (4.0–7.0) (Gilbert et al., 1991). Lipase activity from Aspergillus niger MTCC 2594 was maximum at pH 6.5 (Kamini et al., 1998) but differed from those from A. niger NCIM 1207 (Mahadik et al., 2002) and from A. carneus (Saxena et al., 2003) in that the latter were maximum at pH 2.5 and 9.0, respectively.
8.2.3 Agitation vs static condition

Enzyme production by the two isolates was studied for growth under static conditions or with agitation (120 rpm). The results clearly indicated superior lipase production under agitation (Fig 8.4). For all further experiments, isolates were grown under agitation for the production of lipase.

Aeration has variable effects on lipase production by different organisms. The degree of aeration appears to be critical in some cases since shallow layer cultures (moderate aeration) produced much more lipase than shake cultures (high aeration). Vigorous aeration greatly reduced lipase production by *Rhizopus oligosporus*, *Penicillium fragi*, *Pseudomonas aeruginosa* and *Mucor racemosus* (Gilbert *et al.*, 1991). However, high aeration was needed for eliciting high lipase activity by *Penicillium mephitica* var. *lipolytica*, *Aspergillus wentii* and *Mucor hiemalis*. Increasing aeration by shaking initially resulted in both increased growth and lipase production, followed by a rapid decrease of lipase activity as shaking continued (Saxena *et al.*, 1999). In contrast, Oso (1978) reported that stationary conditions in *Torulopsis emersonii* favored maximum lipase production.

8.2.4 Temperature

The effect of temperature on lipase production in culture was studied in the temperature range of 28-50°C as well as at 4°C. From Fig.8.5 it can be observed that the maximum production was at 30 ± 2°C by both isolates AH-2 and TZ. The isolates could neither grow nor produce lipases beyond 45°C.
Fig. 8.3 Effect of pH on alkaline lipase production

Production conditions - MV medium containing olive oil (0.5%), varying pH ranging from 3.5-10, 30 ± 2°C for 96 h under agitation.

Fig. 8.4 Effect of static or agitation conditions

Production conditions - MV medium containing olive oil (0.5%) pH 6.0, 30 ± 2°C for 96 h under agitation or static culture, as indicated.
Interestingly, a low but not insignificant activity was elicited when grown at 4°C also. For all further experiments, a temperature of 30 ± 2°C was maintained for the production medium.

The influence of temperature on the production of lipases by fungi has not been extensively studied although temperature appears to be a crucial parameter. For example, for *A. niger* the optimum temperature for lipase production was 24°C and differences as little as 1°C could considerably decrease the yield (Ohnishi *et al.*, 1994). Cultures for production of lipases by fungi of the genus *Penicillium* are generally incubated between 25 and 30°C, most often at 28 °C (Lima *et al.*, 2003). Oso (1978) determined 45°C to be the best temperature for lipase production by *T. emersonii*. A broader temperatures range of 22–35°C was however reported to be conducive for maximum lipase production by *A. wentii* (Chander *et al.*, 1981), *M. heimalis* (Akhtar *et al.*, 1980), *R. nigricans* (Chander *et al.*, 1981) and *M. racemosus* (Chopra *et al.*, 1981).

### 8.2.5 Effect of crude salt concentration

MV broth with various concentrations of crude salt, 0.5% olive oil and was used as growth medium and the enzyme production was analyzed. The results (Fig 8.6) showed that a crude salt concentration of 3.4% resulted in maximum enzyme production although higher concentrations were also not very inhibitory. These results clearly suggested the prominent role of extracellular lipases in ecological sustenance of these organisms. Garcia *et al.* (1991) reported that maximum lipase activity in *A. niger*, occurred at an optimal salt
Fig. 8.5 Effect of temperature on production of alkaline lipases
Production conditions - MV medium containing olive oil (0.5%) pH 6.0, temperature range $30 \pm 2^\circ C$-$50^\circ C$ for 96 h under agitation.

Fig. 8.6 Effect of crude salt concentration
Production conditions - MV medium at various crude salt concentrations (1-10%) containing olive oil (0.5%) pH 6.0, $30 \pm 2^\circ C$ for 96 h under agitation.
concentration of 2 mM and pH 7.5. Lee and Rhee (1993) and Toida et al. (1998) reported a similar salt requirement in the lipase production medium of \textit{P. Putida} and \textit{A.oryzae}. For all further experiments, a crude salt concentration of 3.4% was maintained for the production medium.

8.2.6 Effect of inducers

Extracellular lipase production by different microorganisms in medium supplemented with lipids has been extensively reported. It was demonstrated that lipase activity is induced by the presence of lipid substrates in the medium (Nutan \textit{et al.}, 2002). The effect on enzyme production of various oils (such as groundnut, sunflower, coconut, palm, gingelly and olive, at 0.5% concentration) introduced as inducers was therefore studied and the results are presented in Fig.8.7. Olive oil at 0.5% concentration was found to be the best inducer for the production of lipase by both the thraustochytrids.

Although all the inducers tested resulted in enzyme production to varying extents, olive oil gave the maximum yield followed by sunflower, coconut, palm, gingelly and groundnut oils. Induction appears to be influenced not only by the lengths of fatty acids in these oils but also by the number of unsaturations. The manner by which these compounds influence lipase biosynthesis is yet not well understood (Lima \textit{et al.}, 2003). The fatty acids present in the greatest proportions in these oils are oleic and linoleic acids. Better lipase production appears to be correlated with a higher content of oleic acid in the oil (Iwai and Tsujisaka, 1984). Among the above oils tested for the production of lipases by both the \textit{Thraustochytrium} sp, olive oil was found to be the best carbon source.
for lipase production probably because of its highest content of oleic acid (28%) and low linoleic acid (3%) while those of other oils contain higher percentages of linoleic acid (~30-50%).

Similar reports on maximum lipase production in presence of inducers at optimal concentration were reported by several workers: in *Pe. Wortmanii* (olive oil) (Costa and Peralta, 1999), in *Aspergillus oryzae* (soyabean oil) and in *Candida rugosa* (olive oil) (Ohnish et al., 1994). Higher oil concentrations could be affecting the aeration rate of the culture and promoting a delay in mycelial growth and lipase production in *A. niger* (Falony et al., 2006).

### 8.2.7 Effect of different media compositions

Various combinations of components of MV medium (as outlined in Section 8.1.6) were used and the enzyme production compared. The results (Fig.8.8) showed that medium no.8 (containing peptone 0.15% and yeast extract 0.01%) was the best and led to maximum enzyme production. Presence of glucose appeared to inhibit the inductive efficiency of olive oil. Enzyme production was same (low) even at lower concentrations of glucose (up to 0.1%). Also increased amounts (up to 0.1%) of added yeast extract concentration did not show any betterment in enzyme production (data not shown).

Many researchers have reported the positive effect of sugars on lipase production in various species of bacteria and fungi (Salleh et al., 1993). In present study, however, sugar substrates only favored the growth of the
Fig. 8.7 Effect of inducers (0.5%) on production of alkaline lipases
Production conditions — MV medium containing various inducers at 0.5% concentration, pH 6.0, 30 ± 2°C for 96 h under agitation.

Fig. 8.8 Effect of different media on production of the alkaline lipases
Production conditions — various combinations of medium components (as in Section 8.1.6) with crude salt (3.4%), pH 6.0, 30 ± 2°C for 96 h under agitation.
microorganism but not the synthesis of lipase by both *Thraustochytrium* sp. Similar results where glucose inhibited lipase production were reported in *Pseudomonas fluorescens* 2D (Makhzoum et al., 1995) and in *P. aurantiogriseum* (Chahinian et al., 2000).

Generally microorganisms provide high yields of lipase when organic nitrogen sources are used. Complex nitrogen sources such as yeast extract, peptones, soybean meal and corn steep liquor have traditionally been used for fungal lipase production (Sharma et al., 2007). For fungi of the genus *Penicillium*, better results have been obtained with organic nitrogen sources or a combination of organic and inorganic sources (such as peptone or yeast extract with ammonium sulfate) than with inorganic compounds (such as ammonium sulfate) as the sole nitrogen source (Pimentel et al., 1994; Freire et al., 1997). Salleh et al., (1993) obtained maximal production of extracellular lipase by the thermophilic fungus *Rhizop. oryzae* when the medium contained peptone as the nitrogen source. At a peptone concentration of 3% (w/v), the highest lipase production was obtained in medium containing 0.5% (v/v) olive oil and a decrease in peptone concentration to half caused a 2.5-fold decrease in lipase activity by *F. oxysporum* (Freire et al., 1997). Thermostable lipase of *Pseudomonas* sp. KW1-56 was produced in a medium that contained peptone (2% w/v) and yeast extract (0.1% w/v) as nitrogen sources (Izumi et al., 1990).

In the present study, media excluding glucose but with higher concentration of peptone (up to 1.0%) were tested, the results (Fig 8.9) showed that 0.5% peptone gave the best enzyme yield.
The harvesting time, pH and shake culture conditions were therefore again optimized with the medium composition now identified (MV medium containing 0.5% peptone, 0.01% yeast extract, 3.4% crude salt and 0.5% olive oil) for maximum enzyme production and the results are shown in Fig 8.10 a, b, c.

The optimum time for harvesting was increased from 4 to 7 days with increase in peptone concentration, while other parameters such as agitation and pH of the production medium remained the same. Iwai and Tsujisaka (1984) had noted that higher nitrogen concentrations typically used to increase lipase production resulted in prolonged growth period by fungi in general. The delayed lipase production (observed at increased peptone concentration) by both *Thraustochytium* spp in cultures appears to be of a similar nature.

In summary, MV medium containing peptone (0.5%), yeast extract (0.01%), crude salt (3.4%) and supplemented with 0.5% olive oil was found to be the most favorable culture conditions for maximum lipase production by both *Thraustochytrium* spp, under conditions of initial pH 6.0 for a time period of 7 days under agitation.
**Fig. 8.9 Effect of peptone concentration**

Production conditions - Medium 8 with varying peptone concentration (0.15-1.0%), olive oil (0.5%) crude salt (3.4%), pH 6.0, 30 ± 2³C for 96 h under agitation.

**Fig. 8.10 a Effect of harvesting time**

Production conditions - Medium with 0.5% peptone, olive oil (0.5%) crude salt (3.4%), pH 6.0, 30 ± 2³C for 10 days under agitation.
Fig. 8.10 b Effect of agitation on production of alkaline lipases
Production conditions: Medium with 0.5% peptone, olive oil (0.5%) crude salt (3.4%), pH 6.0, 30 ± 2°C for 7 days under static conditions or agitation.

Fig. 8.10 c Effect of pH on production of alkaline lipases
Production conditions: Medium with 0.5% peptone, olive oil (0.5%) crude salt (3.4%), pH 3.5-10, 30 ± 2°C for 7 days under agitation.