Chapter 3

Optimization of growth conditions favoring maximum protease production by selected isolates
The effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme by specific compounds. Appropriate cultivation conditions are essential to the successful production of an enzyme and optimization of parameters such as pH, temperature and medium composition is important in developing the cultivation process. Alkaline proteases are generally produced by submerged fermentation. In addition, solid state fermentation techniques have been exploited, albeit to a lesser extent, for the production of these enzymes (Mitra and Chakraborty, 2005; Malathi and Chakraborty, 1991). In commercial practice, the optimization of the medium composition is carried out to maintain a fine balance between the various medium components, thus minimizing the amount of un-utilized components at the end of fermentation. Research efforts have been directed mainly towards evaluating the carbon and nitrogen nutrients, cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation.

In general, no defined medium has been established for the optimum production of alkaline protease from various microbial sources. Each organism or strain has its own special conditions for maximum enzyme production. In our preliminary studies on the development of the production medium, glucose and peptone were found to be important factors in enhancing the alkaline protease production.
From the results presented in the foregoing Chapter on screening and selection of the most proteolytically active thraustochytrids, two isolates TZ and AH-2 have been ear-marked as potent alkaline protease producers. These isolates as identified up to the genus level by Dr. S. Raghukumar, NIO, Goa were found to belong to the genus *Thraustochytrium*. Optimization of growth conditions of the isolates with respect to physico-chemical parameters such as harvesting time, pH, shake vs static conditions, temperature as well as salt concentration and general composition of the culture medium is reported in this Chapter.

### 3.1 Materials and Methods

All chemicals used were of analytical grade and glass double distilled water was used for all preparations. 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.

#### 3.1.1 Harvesting Time

MV Broth (20ml) with 0.5% milk was inoculated with the isolates TZ or AH-2, kept on a rotary shaker at room temperature and the enzyme production was monitored every 24 h up to 96 h.
3.1.2 Growth pH

Flasks containing 20ml of sterile MV Broth with 0.5% milk and at pH (3-11) pre-adjusted using 0.1M HCl / 0.1M NaOH, were inoculated, kept on a shaker at room temperature and the enzyme production was determined after 72 h.

3.1.3 Effect of temperature

MV Broth with 0.5% milk was inoculated with isolates TZ or AH-2, kept under the above optimized conditions but at different temperatures (on a temperature controlled orbital shaker) and the enzyme production was determined.

3.1.4 Effect of crude salt concentration

MV Broth containing 0.5% milk and various concentrations of crude sea salt was used as growth medium to study the enzyme production.

3.1.5 Effect of chemical parameters

Media of different chemical compositions were used for the enzyme production under the above optimum conditions:

1. MV Broth + 0.5% milk
2. Crude sea salt (3.4%) + yeast extract (0.1%) + 0.5% milk
3. Crude sea salt (3.4%) + glucose (0.4%) + 0.5% milk
4. Crude sea salt (3.4%) + peptone (0.15%) + 0.5% milk
5. ASW + glucose (0.4%) + peptone(0.15%) + yeast extract (0.1%) + 0.5% milk
6. ASW + 0.5% milk
Unless otherwise mentioned, concentrations of specific medium components were the same as routinely used in MV medium (*vide* Appendix I). Composition of ASW (artificial sea water) is also given in Appendix I.

### 3.2 Results and Discussion

The various physical and chemical parameters of the production medium studied for maximum protease production were harvesting time, pH, agitation vs static conditions, temperature and concentration of crude salt besides composition of the medium.

#### 3.2.1 Time course of enzyme production

Protease production was studied for different time periods at room temperature (30 ± 2°C) in MV medium supplemented with 0.5% skimmed milk powder. The results showed that the protease production by both the isolates AH-2 and TZ was initiated after 24 h, increased consistently with time and reached a maximum after 72 h (Fig 3.1). In a separate experiment (data not shown) protease production was found to peak during the stationary phase of growth.

So for all further experiments, time period of 72 h was chosen for maximum enzyme production by both isolates.

Similar results were obtained by Dutta and Banerjee (2006) while studying the physical parameters affecting protease production by *Pseudomonas* sp. RAJR 044. The severe drop in activity after three days in culture was attributed to exhaustion of nutrients. Maximum protease production by *Bacillus* sp. K-30 was recorded after 96 h of incubation at 50°C (Naidu and Devi, 2005) and by the
fungus *Mucor circinelloides* at the end of 96 h of incubation (Andrade et al., 2002). In contrast, protease activity of a yeast strain, *Aureobasidium pullulans*, reached maximum within 30 h of the fermentation when the cell growth reached mid log phase (Chi *et al.*, 2007).

### 3.2.2 Growth pH

An important characteristic of most microorganisms has been noted to be their strong dependence on the extracellular pH for cell growth and enzyme production (Kumar and Tagaki, 1999). The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Majority of the thermophilic bacilli are found to grow at pH and temperature ranges of 5.8-8.0 and 50-65°C, respectively (Zeikus, 1979).

In the present work, the production medium was adjusted to varying pH ranging from 3.5-10 and the effect on protease production is presented in Fig.3.2. Although maximum enzyme production by the thraustochytrids was found to occur during growth at pH 7.0 for both isolates, the activity of the enzyme from isolate TZ was approximately 68% more than that from isolate AH-2. For all further experiments, a pH of 7.0 was maintained for the production medium.

Alkaline protease production by *Bacillus* spp. is found to be maximum at pH 9-13 (Borris, 1987). Chi *et al.* (2007) have reported that the yeast strain *Aureobasidium pullulans* produced the highest yields of alkaline protease when the initial pH of the production medium was 7.0. Protease production by
Fig. 3.1 Time course of alkaline protease production

The isolates were grown in MV medium supplemented with 0.5% skimmed milk powder for 5 days and the protease activity was determined at regular intervals of 24 h.

Fig. 3.2 Effect of pH on the production of alkaline proteases

Production conditions - MV medium containing skimmed milk powder (0.5%) varying pH ranging from 3.5-10, after 72 h under agitation of 120 rpm at 30 ± 2°C.
Pseudomonas sp. RAJR 044 was maximum at pH 6.0 (Dutta and Banerjee, 2006). Aunstrup (1980) had reported that in general, for increased protease yields from alkalophilic microorganisms, the pH of the medium must be maintained above 7.5 throughout the fermentation period. As the metabolic activities of the microorganisms were very sensitive to pH change, protease production was affected if pH level was higher or lower than the optimum value (Tunga et al., 1998; Ali and Roushyd, 1998).

3.2.3 Agitation

Alkaline proteases are generally produced by submerged fermentation. During the fermentation, different levels of dissolved oxygen in the fermentation broth can be obtained by variations in the agitation speed. This can influence greatly cell growth of the microbes and thereby production of extracellular enzymes (Chi and Zhao, 2003).

When protease production was studied under static and shake flask conditions (120 rpm) for 72 h at room temperature, the enzyme production by both the Thraustochytrium sp was found to be significantly high under agitation in relation to static conditions of culture (Fig.3.3). Chi et al. (2007) had also observed that an agitation speed 150 rpm was most suitable for protease production by the yeast strain Aureobasidium pullulans and by Virgibacillus pantothenticus (Gupta et al., 2007). For all further experiments, isolates were grown under agitation for the production of protease.
3.2.4 Temperature

When grown in medium at various temperatures, it was observed that maximum production of alkaline protease occurred at 30 ± 2°C (Fig.3.4.) for both the thraustochytrid isolates. The isolate TZ showed 76 % more activity than AH-2. Both the isolates could not grow at temperatures beyond 45°C and hence there was no enzyme production either at these temperatures. For all further experiments, a temperature of 30 ± 2°C was maintained for the production medium.

Temperature is understandably one of the most critical parameters that has to be controlled in bioprocess (Chi and Zhao, 2003). Their results on temperature effects have revealed that the specific protease activity of the yeast strain *Aureobasidium pullulans* reached the highest when grown at 24.5°C (Chi *et al.*, 2007). Generally, 30°C has been the optimum temperature reported for maximum secretion of extracellular protease. Higher temperature was found to have adverse effect on the metabolic activities of the microorganism (Tunga *et al.*, 1998; Singh and Vyas, 1975).

3.2.5 Effect of crude salt concentration

When MV Broth containing 0.5% milk and various crude salt concentrations was used as the growth medium, both the isolates AH-2 and TZ were found to be able to grow over a wide range of salt concentrations (1-10%, w/v). A concentration of 3.4% crude salt resulted in maximum enzyme production although concentrations as high as 6% also elicited as much as 89 and 53 % of
Fig. 3.3 Effect of static or agitation conditions

Production conditions - MV medium containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2°C for 72 h under shake or static culture.

Fig. 3.4 Effect of temperature on alkaline protease production

Production conditions - MV medium containing skimmed milk powder (0.5%) pH 7.0, at different temperatures for 72 hours under agitation.
the maximal activity for the isolates TZ and AH-2, respectively (Fig.3.5). These results are in tune with the halotolerant nature of these thraustochytrids.

Studies by Mital et al. (2006) on the salt requirement by a group of 8 moderately halophilic and alkaliphilic bacteria for optimum enzyme secretion revealed that the enzyme production varied significantly among the isolates. The salt dependency was however, relatively lower when compared to that of extremophilic isolates such as the haloalkaliphilic Archaean ones from Soda Lake where maximum growth was at 10% salt (w/v) while for maximum protease production 15% salt was required (Sorokin et al., 2003).

Similar results have also been found for the moderately halophilic and alkaliphilic coccus Salinicoccus alkaliphilus sp. isolated from Baer Soda Lake in Mongolia, which could grow over a range of 0-25% (w/v) NaCl with an optimum at 10% (w/v) for best growth and enzyme secretion (Zhang et al., 2003).

3.2.6 Effect of chemical parameters

Investigations on protease production by many fungal cultures have shown that there were drastic variations with the composition of the medium used. For instance, the alkaline protease production by Mucor circinelloides was shown to be predominantly influenced by glucose and peptone concentrations (Andrade et al., 2002).
When medium of different compositions was used to study the enzyme production under the above derived optimum conditions, it was seen that MV broth with 0.5% milk was the best for the alkaline protease production by both the *Thraustochytrium* spp. (Fig.3.6). Omission of any one of the ingredients resulted in poor enzyme production. Although ASW supported good growth (data not shown) it failed to elicit production of alkaline protease.

It was observed that the lack of glucose resulted in a dramatic decrease in enzyme production by both the *Thraustochytrium* spp. Glucose has been reported to suppress protease production in *Bacillus licheniformis* (Sen and Satyanarayana, 1993; Sonnleitner, 1983) but in the present study it was found to be a relatively good substrate component for enzyme production, especially when used at low concentrations (0.4%). Other workers have also reported better protease production in the presence of glucose as a substrate (Gajju, 1996).

The protease production by *Yersinia ruckeri* was influenced by the composition of the culture medium; protease production were optimum in peptone medium whereas activity was not detected when the microorganism was grown in the presence of Casamino Acids, suggesting that intact peptides are necessary for the induction process (Secades and Guijarro, 1999). A similar behavior has been observed for *Aeromonas salmonicida* (Dalhe, 1971), *Aeromonas liquefaciens* (Braun and Schmitz, 1980), *Vibrio* species (Dreisbach and Merkel, 1978) and *E. chrysanthemi* (O'Reilly and Day, 1983).
Fig. 3.5 Effect of crude salt concentration

Production conditions - MV medium at various crude salt concentrations (1-10%) and containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2°C and agitation under 72 h.

Fig. 3.6 Effect of medium composition

Production conditions – Medium containing various combinations of skimmed milk powder (0.5%) with different media components maintained at pH 7.0, 30 ± 2°C for 72 h under agitation.
The effect on protease production by our isolates of varying concentrations of glucose, peptone and yeast extract (present in MV medium) has been studied. The enzyme production was increased from 8.5 to 30 and 3.6 to 20.5 U/ml respectively, for the isolates TZ and AH-2, with an increase in glucose concentration from 0.2 to 0.4 % (w/v). Beyond 0.6 %, however, the enzyme production was highly suppressed (Fig.3.7a) suggesting a threshold level of glucose for optimum protease production. A similar relationship was reported in case of an alkaline protease from Yersinia ruckeri (Secedes and Guijarro, 1999) and alkaliphilic Bacillus sp. (Patel, 2006) where glucose repressed protease beyond the optimum level. A catabolic repression mechanism for extracellular enzyme production has been described for V. alginolyticus, Pseudomonas maltophilia and Staphylococcus aureus, suggesting that in the absence of the sugar (i.e., glucose) the protease plays a role in supplying peptides or amino acids as the carbon or energy source in addition to providing the nitrogen source.

Protease synthesis may hence be repressed when the energy status of the cells is high. This kind of regulatory mechanism has been postulated for proteases of other pathogens such as P. aeruginosa and Vibrio strain SA1 (Secedes and Guijarro, 1999).

Peptone and yeast extract enhanced the enzyme production when included in medium. The optimum peptone and yeast extract concentrations for maximum protease production were found to be 0.1 to 0.15 % for both the Thraustochytrium spp. Further increase beyond the optimum level did not
cause much change in enzyme yield from isolate TZ but resulted in decrease in the enzyme production from isolate AH-2 (Fig 3.7 b & c). A combination of carbon (glucose) and nitrogen sources (peptone and yeast extract) thus resulted in a maximum enzyme production by both Thraustochytrium spp. Patil et al. (2006) have reported similar requirement of peptone (0.5%), yeast extract (0.5%) and glucose (1.0%) for maximum alkaline protease production by Bacillus sp..

In summary, the optimized culture conditions for maximum production of alkaline protease by both the isolates AH-2 and TZ were a temperature of 30 ± 2°C and pH 7.0 in the presence of 0.5% skimmed milk as an inducer, when grown for 72 h under agitation. Peptone (0.15%), yeast extract (0.1%) and glucose (0.4%) elicited best alkaline protease production in both the isolates.

Although the requirements of culture conditions were similar, the isolate TZ exhibited more enzymatic activity than isolate AH-2. It is noteworthy to mention that a simple and cost-effective medium has been used for the alkaline protease production by both Thraustochytrium spp.
Fig. 3.7a Effect of glucose concentration

Production conditions - MV medium at various glucose concentrations containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2°C for 72 h under agitation.

Fig. 3.7b Effect of peptone concentration

Production conditions - MV medium (at glucose 0.4%) and various peptone concentrations, containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2°C for 72 h under agitation.
Fig. 3.7c Effect of yeast extract concentration

Production conditions - MV medium glucose 0.4 % & peptone 0.15 %) at various yeast extract concentrations and containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2°C for 72 h under agitation.