Production of alkaline protease under submerged fermentation

The use of microorganisms for the production of commercially important metabolites has increased rapidly over the past half century and the production of enzymes in submerged fermentation (SmF) has long been established. A significant number of highly economical SmF processes are known to exist not only for enzyme production but also for the production of several other metabolites and are being successfully exploited by industry in most countries of the world. Extensive investigations have accomplished great developments in the technology overriding the earlier shortcomings due to unoptimised preculture and other growth conditions.

Submerged fermentation involves the growth of microbes on solid substrates dissolved or submerged in liquid. The limited availability of water makes SSF quite different from SmF. Compared to solid state fermentation, very little information is available on submerged state fermentation. The
advantages of SSF for protein enrichment and bioconversion of substrates over submerged fermentation are very clear and significant. This has led to SmF being utilised exclusively for enzyme production especially by fungal cultures. Most investigations on enzyme production methods are concerned with producing fungal or bacterial biomass in submerged cultures. These culture methods permit better control of environmental factors such as temperature and pH.

Implementation of submerged fermentation for the production of various secondary metabolites was described by several authors. Bartholomew et al. (1950) described the mass transfer of oxygen in submerged fermentation of *Streptomyces griseus*. The nature and quantity of by-products formed during conversion of sugar beets to ethanol by *Zymomonas mobilis* in conventional submerged fermentation were investigated by Amin and Allah (1992). Morphology and citric acid production of *Aspergillus niger* PM1 in submerged fermentation has been worked out by Papagianni (1995). Papagianni et al. (2001) have made a successful attempt to produce phytase under submerged fermentation using *Aspergillus niger*. Ghildyal et al. (1993) compared the economics of submerged and solid state fermentation for the production of amyloglycosidase. Senecal et al. (1992) reported that SmF was favoured over SSF in biotransformation of corn stover when used as a carbon source. Crestini et al. (1996) described a method for the production and isolation of chitosan by liquid and solid state fermentation from *Lentinus edodes*.

A general opinion about the choice of fermentation method for the production of any microbial product would normally be SmF, unless there appears a particular reason why SSF should be chosen. It is a familiar technique, scale-up from laboratory level to industry is much simplified, with parameters being more easily monitored and controlled. However, there are
problems associated with secondary metabolite production in liquid fermentation such as shear forces, increasing viscosity due to metabolic secretion and reduction in metabolic stability. However, SmF is intrinsically less problematic – heat transfer is better, and homogeneity is maintained. In fact, SmF has many features which would make it the preferred method in cases where SmF and SSF had similar economic performances.

One of the differences between solid-state and submerged cultures is that in the former, the moisture content of the substrate is low, resulting in a limitation of growth and metabolism of the microorganism. The concept of water availability in a substrate, thus, becomes very important. This difference is one of the crucial factors that govern the processes that occur during fermentation. Moisture content being related to many factors can greatly influence the path of enrichment, leading to products that differ both quantitatively and qualitatively.

Several reports are available on enzyme production by SmF. The strain *A. oryzae* NRRL 2160 was reported to produce high levels of extracellular protease (Dworshack, 1952). Klapper et al. (1973) reported the production and purification of a serine protease from this strain in submerged culture. Proteases from *Aspergillus oryzae* have been produced in submerged cultures (Nakadai and Nasuno 1988; Fukushima et al., 1989). Comparatively higher lipid enrichment of prawn shell waste in SmF by various protease producing bacterial strains was described by Amar (2001).

The requirement of water for growth and metabolic activities of microorganisms and the consequent potential of the water activity of the medium in controlling biotechnological processes are well established (Hahn-Hagerdal, 1986). Thus it is expected that the increase in moisture content of the medium would lead to increased productivity of the system. It was only recently, that it became apparent that the biochemical and physiological
responses of many organisms in solid state culture differ greatly from those in SmF, leading to variations in productivity. It is also well known that the strains isolated and developed for the use in SSF processes are poor producers of enzyme in the SmF system and vice versa. So it is imperative to study the preferences of the selected strain to obtain optimal performance.

3.1 Materials and methods

3.1.1 Inoculum preparation

Inoculum was prepared as described in section 2.1.2

3.1.2 Alkaline protease production under SmF

The SmF experiments were carried out in 250 ml Erlenmeyer flasks using 1.25g of the substrate submerged in 25ml of 0.2M carbonate/bicarbonate buffer (pH 9.2). The medium was autoclaved at 121°C, 15lb pressure for 15min, cooled to room temperature and inoculated with 80% (v/w) inoculum (5x10⁸ cells/ml). The cultures were then incubated at 37°C and 100rpm for 120 h and samples were withdrawn for enzyme assay. Plate 3 shows submerged fermentation set up in a 250 ml conical flask.

3.1.3 Enzyme extraction

After fermentation the whole contents were filtered and squeezed through a muslin cloth. The clear extract obtained after centrifugation was assayed for proteolytic activity.

3.1.4 Alkaline protease assay

Alkaline protease was assayed as described in section 2.1.5

3.1.5 Optimisation of fermentation process parameters under SmF

The strategy followed was to optimise each parameter, independent of the others and subsequently optimal conditions were employed in all the
SmF set up in a conical flask

Plate 3
Production of alkaline protease under submerged fermentation experiments. The various process parameters optimised for maximal protease production were as follows:

3.1.5.1 Effect of different substrates on protease production

All the substrates experimented for the production of protease under SSF (2.2.6.1) were tried in SmF as well. The ideal substrate obtained was used for subsequent experiments.

3.1.5.2 Effect of incubation period on protease production

The culture medium was incubated for varying periods of time, 24, 48, 72, 96 and 120h to find the optimum time required for maximum enzyme production.

3.1.5.3 Effect of inoculum size on protease production

The influence of inoculum size on protease production was studied by altering the inoculum levels as 20, 40, 60, 80 and 100 (%v/w) in the medium.

3.1.5.4 Effect of temperature on protease production

Fermentation was carried out at various temperatures such as 20°C, 25°C, 30°C, 37°C and 45°C to evaluate their influence on protease production. The experiments that followed were conducted at the temperature optimised at this step.

3.1.5.5 Effect of amount of substrate on protease production

Different quantities of substrate (%w/v) 5, 10, 15 and 20 were submerged in the fermentation medium to investigate the influence of the amount of substrate on the magnitude of enzyme production. The minimum quantity of substrate yielding maximum enzyme was selected for further experiments.
3.1.5.6 Effect of particle size of substrate on protease production

The substrate was sieved through various mesh size to obtain fine, medium, coarse and large particles of < 425 μm, 425-600 μm, 600-1000 μm and 1000-1425 μm size respectively to see whether the particle size has any influence on enzyme production.

3.1.5.7 Effect of NaCl concentration on protease production

The effect of sodium chloride was evaluated by incorporating it at various concentrations of 0, 0.05, 0.1, 0.2 and 0.4 (%w/w) in the fermentation medium.

3.1.5.8 Effect of supplementary carbon sources on protease production

The requirement of additional nutrient supply was studied, adding different supplementary carbon sources (10%w/w) like glucose, sucrose, maltose, lactose, glycerol, starch, mannose and molasses to the fermentation medium. The ideal carbon source thus obtained was included in the medium formulation to continue the optimisation studies of the remaining parameters.

3.1.5.9 Effect of supplementary nitrogen sources on protease production

Whether the addition of supplementary nitrogen sources could enhance the production of protease was studied by supplying organic sources like beef extract, casein, gelatin, peptone, tryptone, yeast extract and corn steep liquor; and inorganic nitrogen sources like NaNO₃, NH₄Cl, (NH₄)₂SO₄, NH₄NO₃ and urea at a level of 10% w/w in the medium.

3.1.5.10 Effect of shaking speed on protease production

The effect of aeration and mixing of substrate on protease production under SmF was determined by incubating the culture medium at different rpm (0, 50, 100, 150 and 200) in a rotary shaker.
All the experiments were carried out in triplicates in order to corroborate the validity of the results obtained.

3.2 Statistical analysis

Data generated from the above experiments were analysed by One-way Analysis of Variance (ANOVA). Mean of the results was compared using SPSS 10.0 for windows at a significance level of $p<0.05$.

3.3 Results and Discussion

The results of the optimisation studies of the fermentation process parameters under SmF are shown as graphs and tables. The ANOVA results are also given in the corresponding tables as superscript alphabets. Values with the same superscripts do not vary significantly.

3.3.1 Optimisation of fermentation process parameters under SmF

Extracellular protease production in microorganisms is strongly influenced by media components such as, variation in C/N ratio, presence of some easily metabolisable sugars, such as glucose and metal ions. Protease synthesis is also affected by the presence of rapidly metabolisable nitrogen sources in the medium. Besides, several physical factors such as aeration, inoculum density, pH, temperature and period of incubation also affect the amount of protease produced (Hameed et al., 1999; Puri et al., 2002). In the present study also, culture conditions have been found to influence the quantity of protease produced by the selected strain.

3.3.1.1 Effect of different substrates on protease production under SmF

Studies on the effect of different substrates on protease production have shown that different substrates performed quite differently from that of SSF, except for wheat bran, which proved to be the best substrate in SmF as well. Rice bran and ragi which yielded very low quantities of enzyme in SSF
produced good results in SmF while rawa and maize bran which gave good results in SSF were poor producers in SmF. The order of substrate suitability as per the ANOVA results was wheat bran > ragi > rice bran > black gram bran > soyabean bran, rawa, maize bran and bread powder > cassava > barley (Fig. 3.1). Wheat bran being the best source for enzyme production was used in the subsequent experiments.

![Graph showing enzyme production from different substrates](image)

**Fig. 3.1 Effect of different substrates on protease production: SmF**

The present study makes it evident that the choice of substrate is equally important as the producer organism for successful fermentation. Decreased moisture content might have impaired the accessibility of rice and ragi to the microorganism in SSF. With free flowing water in the medium, these substrates yielded higher amounts of enzyme whereas rawa and maize bran favoured low levels of water in the medium as demonstrated by better performance under SSF conditions. Wheat bran is suitable for both SSF and SmF with better productivity under SmF which itself indicates that the microorganism under study prefers higher water content in the medium.
Although the moist solids are constantly degraded in nature by microorganisms, very little information is available on the factors affecting the utilisation of solid substrates. In general, growth is attributed to the action of enzymes in breaking down the solids to the components that are capable of permeating into the microbial cells. The mechanisms of uptake of non-permeating substrates such as chitin (Stolp and Starr, 1965), lignins (Ogelsby et al., 1967), keratin (Martin and So, 1969), collagen (Seifter and Harper, 1970), sulphur (Beebe and Umbreit, 1971), proteins (Costerton et al., 1974), metal sulphides (Tuovinen and Kelly, 1974), hydrocarbons (Wodzinski and Coyle, 1974; Velankar et al., 1975), agar (Day and Yaphe, 1975), granular starch (Dunlap et al., 1976) and cellulose (Nesse et al., 1977; Berg and Patterson, 1977) were investigated under conditions of submerged culture.

Partial hydrolysis of the pre-treated solids during fermentation occurs outside the cells due to extracellular or cell wall bound enzymes, although detachment of the cell wall bound enzymes or the release of intracellular enzymes due to cell autolysis may also play an important role (Pollock, 1962; Knapp and Howell, 1980). Uptake of moist solids by microorganisms will be influenced by various physical and chemical factors such as the shape of solid particles, porosity, particle size, fibrousness, surface-to-mass ratio, crystallinity, amorphism, stickiness, diffusivity within the solids, mass transfer to and from the solids, degree of polymerisation, hydrophobic or hydrophilic nature, surface electrochemical properties and chemical properties decisive in allowing adsorption of microorganisms on the substrate surfaces (Lonsane et al., 1985). The microbial modification of the substrate resulting in partial hydrolysis is also governed by a variety of factors such as production of enzymes by the cells, uptake of permeable products, diffusion of enzyme into mass of solids, accessibility of solids to cells, heterogeneity of solid surfaces,
organism proximity, requirement of the presence of more readily degradable carbon sources in the system and overall enzyme kinetics (Erickson, 1978).

3.3.1.2 Effect of incubation period on protease production under SmF

Enzyme assay results showed that maximum protease production could be attained at a shorter time in SmF than SSF. A period of 48 h of incubation was enough for maximum yield in SmF compared to 96 h in SSF (Fig.3.2). ANOVA results also substantiated the same results.

An optimal incubation period for maximum protease production is characteristic of the microorganism involved and is largely based on growth characteristics. Bacteria when grown in batch cultures exhibit a characteristic growth curve involving phases of lag, log, stationary and decline, and the phase at which protease production begins in the cell is determined by measuring enzyme production at different time intervals, after inoculation. In the present study, enzyme production begins at the logarithmic phase of growth, reaching the maximal level at the stationary phase of growth. Maximum yield was obtained at 48 h which must apparently be the stationary
and thereafter, there was a decline in the production which can be related to the death phase of the growth curve. The decline in enzyme activity might be due to denaturation and/or decomposition of protease as a result of interactions with other compounds in the fermented medium (Cui et al., 1998).

In a similar study, the protease production in Pseudomonas 1-6 was reported (Sakata et al., 1977) to be very high in the logarithmic phase and then decreased. In some instances, there is little or no enzyme production during the exponential growth phase (Frankena et al., 1985). In a number of cases, the synthesis and secretion of proteases was initiated during the exponential growth phase with a substantial increase near the end of the growth phase and with maximum amounts of protease produced in the stationary growth phase (Durham et al., 1987; Tsai et al., 1988; Takii et al., 1990; Moon and Parulekar, 1991; Ferrero et al., 1996; Manachini et al., 1998).

In SmF maximum production was attained at a shorter time than in SSF. The exclusive features of availability of excess water in the medium and agitation in SmF might be the reason. Vecht-Lifshitz et al. (1990) observed that viscosity of the medium influenced the process parameters in submerged fermentation.

3.3.1.3 Effect of inoculum size on protease production

Higher inoculum levels gave greater yields until a level of 80 % (v/w) beyond which there was no increase in the amount of enzyme produced (Fig.3.3), the pattern being the same as that of SSF.
3.3.1.4 Effect of temperature on protease production under SmF

The temperature preference of *Vibrio* sp. V26 was the same as that of SSF. All the temperatures selected for study supported growth and enzyme production except at 45°C. The optimum was found to be 30°C. Production declined sharply when the temperature of incubation was higher (Fig.3.4).
Optimum temperature for enzyme production is largely characteristic of the organism irrespective of the type of solid support involved (Chandrashekar et al., 1991; Nagendra and Chandrashekar, 1996). In the present study, it was found that the optimal temperature was the same in both SmF and SSF.

3.3.1.5 Effect of amount of substrate on protease production

The quantity of substrate submerged in the medium influenced the enzyme production significantly. There was an increase in the protease yield when the substrate concentration was raised to 10% w/v. This was found to be the optimum as there was considerable reduction in the yield when the substrate concentration was increased further as depicted in Fig. 3.5.

Under SmF conditions, cultures are exposed to hydrodynamic forces (Papagianni et al., 2001) and the amount of substrate in the medium affects the free flow of water in the medium. There was considerable reduction in the yield when the substrate concentration was increased beyond the optimum, may be due to restricted mobility of the substrate in the medium. There is...
scarcely any report available on the optimisation of the amount of substrate under SmF but its impact in the present study was reasonable.

3.3.1.6 Effect of particle size of substrate on protease production

The influence of particle size of the substrate on the production of protease under SmF appeared to be very much similar to that of SSF. Maximum protease production was obtained with substrate particles of average size < 425 μm. Fig. 3.6 shows the results obtained when fermentation was carried out with different particle sizes of the substrate of wheat bran. Larger particles exhibited a decreasing trend towards enzyme yield according to the ANOVA results shown below.

The experimental results showed that the effect of particle size of substrate did not vary with SmF and SSF.

3.3.1.7 Effect of NaCl concentration on protease production under SmF

The NaCl requirement of Vibrio sp. V26 for maximal yield of protease production under SmF conditions is shown in Fig. 3.7. Addition of NaCl in the medium was found to be favourable for improving protease production. A
concentration of 0.2% of NaCl was optimum beyond which there was no increase in production levels. However, there were no considerable variations in the enzyme production at various concentrations of NaCl.

![Graph showing effect of NaCl concentration on protease production: SmF](image)

**Fig. 3.7 Effect of NaCl concentration on protease production: SmF**

The NaCl requirement is an inherent or acquired character of the organism and does not vary significantly with small changes in the fermentation system as is evident from the results obtained in both SSF and SmF.

3.3.1.8 Effect of supplementary carbon sources on protease production

*Vibrio* sp. V26 exhibited the same pattern of preference for carbon sources in SmF as that of SSF. Maltose was found to induce maximum protease production. The yield was found to be much impeded by glucose (Fig. 3.8). ANOVA results showed that maltose was giving significantly higher production followed by molasses, mannose and sucrose.
Wheat bran being rich in most of the primary nutrients required by the bacteria under study, supplementary sources did not have major effects on protease production and hence there is hardly any difference in the choice of these supplements between SmF and SSF.

3.3.1.9 Effect of supplementary nitrogen sources on protease production

The choice of nitrogen sources preferred by the present isolate in SmF and SSF remarkably resembled each other. Unlike inorganic nitrogen sources, the supplement of organic nitrogen sources favoured an increase in protease production. Addition of corn steep liquor marginally increased protease production followed by beef extract and casein. The addition of inorganic sources had an adverse effect on protease production as demonstrated by the reduced enzyme titres. The graphical representation of the results is presented in Fig.3.9.
As described in the previous section, the choice of supplementary nitrogen sources also did not differ from SSF.

3.3.1.10 Effect of shaking speed on protease production under SmF

There was a significant increase in enzyme production when the culture medium was agitated than when it was kept stationary. The production was maximal at shaking speeds above 100 rpm. The results are presented in Fig.3.10.

Fig.3.9 Effect of supplementary nitrogen sources (10% w/w) on protease production: SmF

<table>
<thead>
<tr>
<th>Nitrogen sources</th>
<th>PU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>268.11 ± 0.06</td>
</tr>
<tr>
<td>Casein</td>
<td>268.36 ± 0.12</td>
</tr>
<tr>
<td>Gelatin</td>
<td>267.19 ± 2.21</td>
</tr>
<tr>
<td>Peptone</td>
<td>263.79 ± 0.24</td>
</tr>
<tr>
<td>Tryptone</td>
<td>262.56 ± 1.96</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>265.48 ± 0.41</td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>272.70 ± 0.79</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>121.57 ± 0.56</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>121.72 ± 0.66</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>121.36 ± 0.51</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>121.59 ± 0.51</td>
</tr>
<tr>
<td>Urea</td>
<td>121.65 ± 0.79</td>
</tr>
</tbody>
</table>

Fig.3.10 Effect of supplementary nitrogen sources (10% w/w) on protease production: SmF
For the successful operation of aerobic fermentations, it is essential to supply the microorganisms with sufficient oxygen to meet their requirements at any stage in the process. Failure to supply oxygen may lead to undesirable changes in enzymatic makeup (Rolinson, 1952) or death of the microorganisms (Hromatka et al., 1951). Agitation of the fermenting mass has beneficial effects (Takamine, 1914; Lindenfelser and Ciegler, 1975; Knapp and Howell, 1980) like providing homogeneity through out the fermentation period, promotion of growth on individual particles of the substrate, prevention of aggregate formation, exposure of individual substrate particle to the fermenter's atmosphere, promotion of gas transfer, facilitation of heat exchange, prevention of localised changes and the effective distribution of inoculum.

In the present study, agitation of the medium was found to enhance enzyme production considerably, as it greatly influences the availability of nutrients as well as dissolved oxygen to the organism. After 48 h of incubation, the enzyme production was very low when the culture was kept...
The enzyme production was high at shaking speeds above 100 rpm.

Similar observations on protease production had been made by several workers. In *Vibrio alginolyticus* (Hare et al., 1981) the protease production was markedly influenced by agitation. *Bacillus* sp. B-21-2 produced increased enzyme yields at 600 rpm (Fujiwara and Yamamoto, 1987). Donham et al. (1988) reported 17-fold increase in growth and 7-fold increase in exoprotein production in aerated cultures of *Staphylococcus stimulatus* compared to stationary culture. A high alkaline protease production was reported in a *Bacillus* sp. at 300 rpm (Takami et al., 1989). Similarly, *Bacillus firmus* exhibited maximum production at agitation rate of 360 rpm and aeration rate of 7.0 l min\(^{-1}\). However, lowering the aeration rate to 0.1 l min\(^{-1}\) caused a drastic reduction in the protease yields (Moon and Parulekar, 1991). Optimum yields of alkaline proteases were obtained at 200 rpm for *Bacillus subtilis* ATCC 14416 (Chu et al., 1992) and *Bacillus licheniformis* (Sen and Satyanarayana, 1993). Matta et al. (1994) reported better protease production by *Pseudomonas* sp. AFT-36 under continuous agitation (180 rpm) than that under intermittent or no agitation. In standing cultures protease levels were very low compared to the shaking cultures. The positive effect of aeration was reported by Madan et al. (2000), as aerated cultures gave better protease production than that by stationary culture.

The aeration rates are governed by the nature of the microorganism used, the degree of \(O_2\) requirement for the synthesis of the product, the amount of metabolic heat to be dissipated from the mass, the thickness of the substrate layer employed, the degree to which \(CO_2\) and other volatile metabolites are to be eliminated and the degree of air spaces available in the substrate (Chahal, 1983). For example, a larger gas volume is required by lignocellulosic saprophytic fungi than by the parasitic fungi (Schanel and...
Rypacek, 1958). A higher aeration rate is essential for increased production of aflatoxin, β-galactosidase and invertase (Silman et al., 1979; Silman, 1980; Chahal, 1983) but it inhibits production of achratoxin (Lindenfelser and Ciegler, 1975).

The variation in the agitation speed influences the extent of mixing in the shake flask and will also affect the nutrient availability. This is also one of the means to obtain different dissolved oxygen profiles. During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Therefore, the reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

It has been well demonstrated that the various physico-chemical factors had a profound influence on the alkaline protease production in SmF and maximum production could be obtained by the strategic manoeuvring of the culture medium in accordance with the above results.