CHAPTER 4
ENZYMATIC PROPERTIES OF IMMOBILISED KLEBSIELLA AEROGENES CELLS

Considering the amount of evidence which shows phytic acid to be an antinutritional factor, it is not surprising that a lot of work has been concentrated on the removal of phytic acid from food.

It has long been shown that there is a considerable decrease in the amount of phytate in cereals during the making of bread. As much as 75% reduction in the phytate content of rye bread has been observed (Mollgard et al., 1946). Similarly Harland and Harland (1980) found that there is a reduction of phytate in rye, wheat and whole wheat breads due to fermentation. The extent of reduction is dependent on the initial phytate content. Since the initial phytate content of brown mixes is higher than that of white mixes, there is a lower reduction in phytate content in bread made from the former than that made from the latter (McKenzie-Parnell and Davies, 1986). Expectedly leavening would lead to an increase in mineral availability. Thus, the availability of Zn from leavened bread has been found to be greater than that from unleavened bread (Reinhold et al., 1974). Leavening has also been reported to increase Zn absorption in human volunteers (Naevert et al., 1985). The decrease in phytate content has been attributed to the hydrolytic activity of yeast.
during the leavening process (Reinhold et al., 1974). Chhabra and Sidhu (1988) have reported a considerable hydrolysis of phytate after the addition of yeast and malt to dough. The contribution of two yeast cultures, *Saccharomyces cerevisiae* and *S. minor* to reduction has been evaluated by Bartnik et al. (1987). These were found to have phytase activity while the permanent microflora did not have any activity.

A reduction in phytate content has also been observed during other food fermentations such as soy idli fermentations (Ramakrishnan et al., 1976), tempeh fermentations (Sutardi and Buckle, 1985) and rabadi fermentation (Dhankher and Chauhan, 1987).

The fermentation of various seeds and grains like maize, sorghum, rice, cowpea and soybean have been found to result in as much as 98% reduction in the phytate content. On the other hand, cooking had little reducing effect on phytate levels in whole cereals and legumes (Marfo et al., 1990). The fermentation of pearl millet (*Pennisetum typhoides*) grains by yeast and lactobacilli also brings about a decline in phytate (Khetarpaul and Chauhan, 1989 and 1991). A similar decrease in phytate content is also observed during the fermentation of wheat grains (Gupta et al., 1991 and 1992).

The addition of supplementary phytase to poultry feed has been shown to increase the availability of phytate phosphorus (Nelson et al., 1971). An acetone dried powder of the enzyme prepared from the culture fluids of *Aspergillus ficuum* NRRL 3135
was added to the diet at levels of 3g/1000g. Chickens were able to utilize phosphorus from phytate as efficiently as supplemental inorganic phosphorus. The added phytase was shown to be active in the alimentary tract of the animal and not in the feed prior to ingestion.

The effect of supplementary *A. niger* phytase in diets for pigs has been studied by Jongbloed *et al.*, (1992) and has been shown to increase the digestibility of phytate and the levels of total inorganic phosphorus in the diet as estimated in the ileal and duodenal digests of the animals.

The use of microbial phytase for the improvement of the quality of poultry feed has also been attempted by other workers (*Chang et al.*, 1977; *Liener*, 1977; *Whitaker and Brunnert*, 1977).

The solid state cultivation of *Aspergillus ficuum* in soybean and cotton seed meals has been reported to result in the reduction of phytate content (*Han and Wilfred*, 1988). The application of fungal phytase resulted in the removal of the water insoluble portion of phytate. Prior heating of the substrate (122°C for 1h) and treatment at higher temperature (50°C, pH 4.0 - 4.5) facilitated the hydrolysis of phytate from these substrates. About 85% of phytate in soybean meal was hydrolysed by the microbial phytase whereas only 67% of the phytate in cotton seed meal was destroyed by this treatment.

In a similar study with canola meal, *Nair* and *Duvnjak* (1990) reported a complete reduction of phytate by *A. ficuum* in 48 h.
Older and larger inocula increased the rate of phytate hydrolysis. The crude enzyme produced during this fermentation could be extracted and used for reduction of phytic acid in new batches of canola meal.

Simons et al., (1990) report that the addition of microbial phytase to low phosphorus diets increased the availability of phosphorus to over 60%. The enzyme was active in vitro in soybean meal, maize and a liquid compound feed.

Phytate compounds in various foods and feeds could be dephosphorylated in vitro by non-purified preparations of intracellular acid phosphatase from the waste mycelia of A. niger (Zyla et al., 1989).

The effect of yogurt on the growth of rats fed diets high in phytate has been studied. Animals receiving diets containing phytic acid and mixed with yogurt grew as well as those receiving a diet containing no phytic acid; on the other hand, animals receiving a diet containing phytate and no yogurt had lower weight gains and bone Zn concentrations. The phytate hydrolysing ability of lactobacilli has been implicated in this study (Toleman et al., 1987).

It is known that the germination of seeds results in the reduction of phytate content (Sathe et al., 1983; Lu et al., 1987; Sharma and Sehgal, 1992). The effect of germination of the seeds of mung bean, wheat and chickpeas has been studied in detail by Harmuth-Hoene et al. (1987). While losses of dry matter
and carbohydrate were observed with all three seeds, phytic acid was partially hydrolysed in mung bean and wheat. A decrease in total fat content with a corresponding increase in polyunsaturated fatty acids was also observed. Thus, germination enhances the nutritional quality of seeds both by decreasing the phytate content and by the building up of other nutrients. Kikunaga et al. (1991) report that the germination of rice seeds leads to an increase in phytase activity and an increase in free phosphorus, with a decrease in phytic acid.

The use of physicochemical methods for the removal of phytate, mostly from soy products, has been suggested. Hartman (1979) has suggested a process for the removal of phytate from commercial soy protein isolates containing 2-3% tightly bound phytate. The adjustment of the pH of a water extract of soy flakes to 11.6 at 28°C to precipitate the phytate, removal of this by vacuum filtration or centrifugation, and subsequent neutralization and ultrafiltration resulted in the decrease of phytate content to as low as 0.1%. The protein quality of the purified soy extract was as good as similar acid isolates and commercial soy isolates. Ang et al. (1986) have reported the use of ultrafiltration for phytate removal. 50% of the phytate was found to be removed at 60% water removal. The non-removal of all the phytate was put down to its linking of proteins via salt bridges. In fact, phytic acid is reported to interfere in the extraction of proteins from grain legumes and wheat using acetic acid, due to the formation of insoluble complexes with proteins (Hussain and Buschuk, 1992).
Fretzdorff (1989) has suggested a simple method for the removal of phytate from wheat bran and germ products. By extraction with sodium citrate buffer at pH 5.25 for 0.5 h, the wheat bran was rendered practically free of phytic acid.

There are different reports about the stability of phytate to heating. The autoclaving of various cereal and oil seed products for 30 min failed to decrease the phytate content by even 10% (deBoland et al., 1975). Similarly, various heat treatments - microwave, infrared, hot air oven, autoclaving and cooking in boiling water - had no effect on the phytate of winged beans (Kadam et al., 1987). On the other hand, it is reported by Hussain et al., (1989) that both autoclaving and roasting reduce the phytate content of chickpeas significantly.

Extrusion cooking has variable results. The phytate content of a high fibre cereal product was found to remain unaltered while there was a loss of phytase during cooking (Sandberg et al., 1986). On the other hand, Chauhan et al. (1988) found that there was a reduction in the phytate content of rice legume blends after cooking. The reduction was found to be between 20.3% to 26.8%.

Ullah and Cummins (1987) have reported the immobilisation of fungal phytase and the characterisation of the immobilised enzyme. The immobilised enzyme was able to hydrolyse over 50% of the orthophosphate from phytate upon recirculation for a period in excess of 5.0 h at 25°C.
There have been no reports regarding the use of immobilised cells. Hence it was thought of interest to immobilise whole cells and explore the feasibility of using these in a bioreactor for phytate removal.

MATERIALS AND METHODS

Culture

Cells of *Klebsiella aerogenes* were used in the present study.

Immobilisation of cells

The organism was grown in 4 l of inositol medium (see Appendix I for composition) and the cells were harvested by centrifugation at 25000xg. The cells (14 g wet weight) were then resuspended in 28 ml of normal saline. These cells were then added to 39 ml of the monomer solution of acrylamide and bis-acrylamide (75 g of acrylamide monomer and 4 g NN'-methylene bis-acrylamide dissolved in 240 ml water and kept at 8°C in a brown bottle). 390 mg of ammonium persulphate were used for initiating the polymerisation reaction and a few drops of TEMED were added to accelerate the reaction. The immobilisation was carried out under ice. After gelling, the immobilised cells were made to granules by mechanical disintegration. The immobilised cells were then studied with respect to their enzyme activity.

Measurement of phytase activity

Phytase activity was assayed according to the conditions given in table 4.1.
TABLE 4.1

Conditions for phytase assay

<table>
<thead>
<tr>
<th>Substrate</th>
<th>sodium phytate, 0.4 μmols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>acetate (pH 4.5), 100 μmols</td>
</tr>
<tr>
<td>Free cells</td>
<td>equivalent to 500 μg protein</td>
</tr>
<tr>
<td>Immobilised cells</td>
<td>200 mg</td>
</tr>
<tr>
<td>Total volume</td>
<td>2 ml</td>
</tr>
<tr>
<td>Temperature of incubation</td>
<td>45°C</td>
</tr>
<tr>
<td>Time of incubation</td>
<td>20 min</td>
</tr>
<tr>
<td>Start of reaction</td>
<td>Addition of cells</td>
</tr>
<tr>
<td>Termination of reaction</td>
<td>Addition of 2 ml of 10% TCA</td>
</tr>
<tr>
<td>Treatment of blank</td>
<td>2 ml 10% TCA added to the assay mixture before adding cells</td>
</tr>
<tr>
<td>Parameter measured</td>
<td>P$_1$ by the method of Fiske and Subba Row (1925)</td>
</tr>
<tr>
<td>Enzyme units</td>
<td>μmol of P$_1$ liberated per min under assay conditions</td>
</tr>
</tbody>
</table>

**Protein assay**

Protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Kinetic studies of the immobilised cells

The immobilised cells were characterised to determine the optimum conditions for phytate hydrolysis using these cells. The parameters studied were pH, temperature, buffer, thermal stability, storage stability and pH stability. The same parameters were studied for the crude enzyme and whole cells.

a) Optimum pH

The activity of the immobilised cells was checked at different pH using the buffers given in table 4.2. 200 mg of the immobilised cells were taken for each assay and these were incubated at 45°C with the buffer substrate mixture. The reaction was arrested by the addition of 2 ml of 10% TCA and Pi was estimated. The buffers were used at a final concentration of 50 mM.

<table>
<thead>
<tr>
<th>pH Range</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 to 3.6</td>
<td>Glycine-HCl</td>
</tr>
<tr>
<td>3.6 to 6.0</td>
<td>Acetate</td>
</tr>
<tr>
<td>6.2 to 8.0</td>
<td>Imidazole-HCl</td>
</tr>
<tr>
<td>8.0 to 9.5</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td>9.0 to 10.5</td>
<td>Glycine-NaOH</td>
</tr>
</tbody>
</table>
b) Optimum temperature

Temperatures ranging from 30-75°C were used for the assay of phytase activity of whole cells, immobilised cells and enzyme with an incubation time of 10 min. Acetate buffer, pH 4.6 was used for the assay.

c) Determination of apparent $k_m$

The effect of substrate concentration on the rate of phytase catalysed reaction was studied in the concentration range of 0.05 mM to 4 mM for sodium phytate in acetate buffer at pH 4.6. The $k_m$ was also determined for whole cells, and both the $k_m$ values were compared with each other and with the $k_m$ of the enzyme.

The Michaelis constants ($k_m$) were determined from the Lineweaver - Burk plots, which were made by plotting the reciprocals of the initial velocities against the reciprocals of the respective substrate concentrations.

d) Activity in different buffers

Phytase activity of immobilised cells was assayed using acetate and citrate buffers. 200 mg of immobilised cells were incubated with the buffer-substrate mixture for 10 min. The activity of the immobilised cells was compared with that of free cells and the enzyme. The study was conducted at pH 3.6, 4.0, 4.2, 4.6, 5.0 and 5.5.

Stability studies

1) pH stability

The phytase activity of 200 mg cells was measured at pH 4.6
after a 2 hour preincubation in different buffers at pH ranging from 2.5 to 6.0. The buffers were used at 50 mM concentration.

2) Thermal stability - short term

Thermal stability studies were carried out at temperatures ranging from 30-75°C. The cells, in acetate buffer at pH 4.6, were preincubated at the respective temperatures for 10 min and assayed for phytase activity under the optimum conditions.

3) Thermal stability - long term

Thermal stability of the phytase activity of immobilised cells was studied over a period of 48 h. This was done by incubating the cells in acetate buffer, pH 4.6 at 50°C and 60°C and assaying periodically for activity.

4) Storage stability

Immobilised cells were kept at 10°C and assayed for phytase activity periodically over a period of 50 days.

Column studies

The mixture of substrate, buffer and distilled water was passed through a column of immobilised cells (1.5 x 9.0 cm) of volume 10 ml. A flow rate of 0.5 ml per min was maintained. Sodium phytate, the substrate, was adjusted to 400 μM in 50 mM acetate buffer, pH 4.6. After all the mixture had passed through, it was reloaded on the column to check whether there was an increase in the extent of hydrolysis. All runs were carried out at 30°C. The column was washed with acetate buffer and a fresh substrate-buffer mixture was loaded. This was done for a total of
4 times and the conversion rate measured for every run.

RESULTS

Immobilisation of cells

Polyacrylamide was chosen as the support to immobilise the cells since the gel has the following properties:

1) It is inexpensive
2) It is resistant to biological attack
3) Its mechanical strength can be increased by varying the degree of cross linking.

However, there was considerable loss of activity (80%) on immobilisation. A similar loss has been reported for fungal phytase (Ullah and Cummins, 1987).

Kinetic Studies

1) Optimum pH

There was no shift in the pH optimum on immobilisation. The pH optima was found to be 4.6 for both the free cells and immobilised cells (fig. 4.1). The crude enzyme showed optimum activity at pH 4.6. This is similar to the pH optima of most phytases (table 2.6), which range from 2.2 to 6.0. However, it is lower than the one determined for purified phytase (5.2).

2) Phytase activity of immobilised cells in different buffers

Of the two buffers - acetate and citrate - tried out, the whole cells, enzyme and the immobilised cells showed higher activity in the acetate buffer (table 4.3) and therefore acetate was chosen for the rest of the assays.
Fig. 4.1: Effect of pH on phytase activity

Relative activity (%) vs pH

- Immobilised cells
- Crude enzyme
- Free cells
TABLE 4.3
Phytase activity in different buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Free cells % Conversion</th>
<th>Immobilised cells % Conversion</th>
<th>Enzyme % Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td>4.6</td>
<td>61.8</td>
<td>17.6</td>
<td>60.4</td>
</tr>
<tr>
<td>citrate</td>
<td>4.6</td>
<td>48.3</td>
<td>13.4</td>
<td>30.9</td>
</tr>
</tbody>
</table>

3) Optimum temperature

The variation in activity with a change in the temperature of incubation shows that higher temperatures are preferred for phytate hydrolysis by both immobilised and free cells and the crude enzyme. While whole cells showed a temperature optimum of 45°C, there was a shift of 5°C in apparent temperature optimum of the immobilised cells which were maximally active at 50°C (fig. 4.2). The crude enzyme had an optimum of 60°C.

The shift in optimum temperature, on immobilisation, has also been reported for fungal phytase. A shift from 58°C to 65°C on immobilisation has been reported by Ullah and Cummins (1987).

4) Determination of apparent $k_m$

The apparent $k_m$ of the immobilised cells relative to sodium phytate was 400 µM and was identical to that of the free cells.
Fig. 4.2: Effect of temperature on phytase activity

Relative activity (%)

Temperature (°C)

- Immobilised cells  - Crude enzyme  - Free cells
However, this is higher than the $k_m$ of the crude enzyme which was found to be 145 μM and that of the purified enzyme which was 114 μM. Similarly, a considerable increase in the $k_m$ of fungal phytase, on immobilisation, has been reported by Ullah and Cummins (1987). The $k_m$ was found to change from 40 to 138 μM.

Stability studies

1) pH stability

The immobilised cells were stable over a pH range of 2.5 to 5.5 (fig. 4.3). They were maximally stable at pH 4.6 and retained as much as 60% activity at pH 6 and 70% at pH 2.5.

2) Thermal stability - short term

The immobilised and free cells and the crude enzyme were not stable to heat. While the free cells and the crude enzyme lost activity totally at 70°C and 75°C respectively, the immobilised cells retained 10% activity at 75°C (table 4.4).

In contrast, immobilised fungal phytase is reported to be quite stable, suffering negligible loss in activity after exposure to a temperature of 70°C for 30 min (Ullah and Cummins, 1987).

3) Thermal stability of immobilised cells - long term

The thermal stability was greater at 50°C than at 60°C. 33% of the initial activity was retained after 48 h at 50°C but only 12% of the original activity was retained at 60°C (table 4.5).

4) Storage stability of immobilised cells

The cells were stable and retained 68% of the initial
Fig. 4.3: pH stability of immobilised cells

Relative activity (%)
activity after storage at 10°C for 50 days (Table 4.6). However, these were less stable than immobilised fungal phytase which did not suffer significant loss in activity after storage at 5°C for 45 days (Ullah and Cummins, 1987).

**TABLE 4.4**

Thermal stability of phytase activity - short term

<table>
<thead>
<tr>
<th>Temperatures</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Whole cells</td>
</tr>
<tr>
<td>30</td>
<td>100.0</td>
</tr>
<tr>
<td>35</td>
<td>95.0</td>
</tr>
<tr>
<td>40</td>
<td>87.0</td>
</tr>
<tr>
<td>45</td>
<td>85.0</td>
</tr>
<tr>
<td>50</td>
<td>58.0</td>
</tr>
<tr>
<td>55</td>
<td>50.0</td>
</tr>
<tr>
<td>60</td>
<td>24.0</td>
</tr>
<tr>
<td>65</td>
<td>10.5</td>
</tr>
<tr>
<td>70</td>
<td>0.0</td>
</tr>
<tr>
<td>75</td>
<td>0.0</td>
</tr>
</tbody>
</table>
### TABLE 4.5
**Thermal stability of phytase activity - long term**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>50°C</th>
<th>60°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>87.0</td>
<td>62.0</td>
</tr>
<tr>
<td>4</td>
<td>62.7</td>
<td>60.0</td>
</tr>
<tr>
<td>6</td>
<td>60.0</td>
<td>57.0</td>
</tr>
<tr>
<td>24</td>
<td>40.0</td>
<td>33.0</td>
</tr>
<tr>
<td>48</td>
<td>33.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

### TABLE 4.6
**Storage stability of phytase activity**

<table>
<thead>
<tr>
<th>Days</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>76</td>
</tr>
<tr>
<td>30</td>
<td>69</td>
</tr>
<tr>
<td>50</td>
<td>69</td>
</tr>
</tbody>
</table>
Column studies

The immobilised cells were able to hydrolyse 40% of the total available phosphorus during each column run (table 4.7). Recirculation of the effluent substrate did not increase the extent of hydrolysis. Similar activity was exhibited when fresh substrate was passed through the column. The column could be used at least 5 times without any change in activity (table 4.7).

**TABLE 4.7**

Conversion rate per column run

<table>
<thead>
<tr>
<th>No. of runs</th>
<th>% Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.0</td>
</tr>
<tr>
<td>2</td>
<td>41.6</td>
</tr>
<tr>
<td>3</td>
<td>40.0</td>
</tr>
<tr>
<td>4</td>
<td>39.0</td>
</tr>
<tr>
<td>5</td>
<td>40.0</td>
</tr>
</tbody>
</table>

The results obtained with the immobilised cells compare favourably with those reported for fungal phytase. Fungal phytase is reported to hydrolyse 50.4% of the available phosphorus (Ullah and Cummins, 1987). However, this required recirculation of the substrate for at least seven times. On the other hand, the
immobilised cells required only a single passage of the substrate for 40% hydrolysis. Besides, a concentration of 400 μM substrate was used in the present study, while in the case of fungal phytase, the concentration used was 750 μM.

Summary

There was a considerable loss in activity, on immobilisation (80%). The reason for this could be the lowered accessibility of the substrate to the cells. However, the affinity of the cells for the substrate does not change on immobilisation. Both free and immobilised cells had an identical $k_m$ value (400 μM), which was higher than the one determined for the crude enzyme (145 μM) and the purified enzyme (114 μM).

The pH activity profiles of the free cells, immobilised cells and the crude enzyme were similar with maximum activity being observed at pH 4.6. The immobilised cells were very stable over a pH range of 2.6 to 6.0. The temperature optimum of the cells showed a slight shift of 5°C on immobilisation. Immobilisation imparted a small degree of thermal stability to the cells since the cells retained 10% of the initial activity at 80°C while the free cells lost all activity at 70°C.

The immobilised cells showed promising results during column runs. The extent of hydrolysis was comparable to that of fungal phytase. It is possible that the use of a higher temperature for column runs could result in higher hydrolysis. However, considering the thermolability of the immobilised cells, it would be
preferable to use these at 30°C.

On the other hand, the immobilised cells showed better activity than fungal phytase during column runs. While fungal phytase required at least 8 passages of the substrate, for 50.4% hydrolysis at 750 μM concentration, a single passage was sufficient to achieve 40% hydrolysis, by immobilised cells, of the substrate at 400 μM concentration.

Thus it may be concluded that immobilised cells of *Klebsiella aerogenes* have potential to be exploited in a bioreactor for phytate removal.

REFERENCES


Gupta, M., Khetarpaul, N. and Chauhan, B. M. (1992) Rabadi fermentation of wheat; changes in phytic acid content and in


Simons, P. C. M., Versteegh, H. A. J., Jongbloed, A. W., Kemme,


