IMMOBILIZATION OF ENZYMES & BIOTECHNOLOGICAL PERSPECTIVES

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**INTRODUCTION**

- Immobilized enzymes are the enzymes either covalently bound or adsorbed on to the surface of an insoluble support or incorporated in a gel matrix.

- Immobilized enzymes have restricted movement in space either completely or to a small limited region.

- The first Enzyme Engineering Conference was held at Henniker, New Hampshire, USA in 1971. The main theme of this conference was immobilized enzymes.

- Immobilized enzymes are also called as "water-insoluble enzymes", "trapped-enzymes", "fixed-enzymes" and "matrix-supported enzymes".

- In that conference immobilized enzymes were defined as “enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously”.
For the first time in 1916, Nelson & Griffin reported that invertase enzyme extracted from yeast was adsorbed on charcoal and showed the same activity as native enzyme.

In 1948, Summer found that urease from jack bean became water insoluble on standing in 30% alcohol and sodium chloride for 1 to 2 days at room temperature and the water insoluble urease was active.

In 1953, Grubhofer & Schleith immobilized carboxypeptidase, diastase, pepsin and ribonuclease on diazotized polyaminopolystyrene resin.

In 1949, Micheel and Ewers carried out the immobilization of physiologically active protein.

In 1951, Campbell and his colleagues prepared immobilized antigen by binding albumin to diazotized p-aminobenzyl cellulose.
IMMOBILIZED ENZYMES

Carrier-Bound
- Physically Adsorbed
- Ionic Bound
- Covalent Bound

Cross Linked

Entrapped
- Lattice Type
- Microencapsulated
Enzyme Commission number (EC no.) is 2.4.1.1.

Systematic name is $\alpha$-1,4-glucan, orthophosphate, $\alpha$-glucosyl transferase.

Catalyzes the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate, that is

$$\text{Starch} + \text{Inorganic phosphate} \rightleftharpoons \text{Glucose-1-phosphate}$$
**IMPORTANCE OF STARCH PHOSPHORYLASE**

Plays an important role in starch metabolism in plants

Used for the production of glucose-1-phosphate, a cytostatic compound used in cardio-therapy

Used to estimate inorganic phosphate in serum under the pathological conditions as well as to detect amount of inorganic phosphate pollution in the environment

Are in great demand in industries due to their reusability
REVIEW OF LITERATURE

- **Kumar and Sanwal (1981)** for the first time immobilized starch phosphorylase from mature banana leaf on methylene bis acrylamide and also characterized immobilized enzyme.

- Potato phosphorylase was subsequently immobilized by coupling to an insoluble support through diazonium salts and covalent bonding between the enzyme and Eupergit C (**Szulezynski, 1986**).

- **Zeeman et al. (2004)** showed that plastidial α-glucan phosphorylase from *Arabidopsis* is not required for starch degradation since loss of its activity by T-DNA insertions resulted in no significant change in total accumulation of starch during the day or its remobilization at night. They also showed that it plays a role in the capacity of the leaf lumina to endure a transient water deficit.

- **Chen et al. (2002)** showed regulation of the catalytic behavior of starch phosphorylase from sweet potato roots by proteolysis. They showed the presence of 78 amino acids in the center of the enzyme protein that serves as a signal for rapid degradation.
- **Hsu et al. (2004)** reported purification and characterization of a cytosolic starch phosphorylase from etiolated rice seedlings.

- **Jorgensen et al. (2006)** showed the presence of starch phosphorylase protein in potato tuber juice after starch isolation. They also showed that it constitutes a major protein (nearly 4% of total proteins in the juice).

- **Michiyo et al. (2006)** established a system to produce synthetic amylose from either sucrose or cellobiose using the combined action of α-glucan phosphorylase with sucrose phosphorylase or cellobiose phosphorylase.
Millet variety KB 560 seeds were purchased from Seed Market, Indore.

Seeds were washed with tap water thoroughly and subsequently with distilled water.

Thereafter, these were surface sterilized with 0.1% mercuric chloride solution for 2-3 min.

The surface sterilized seeds were kept for germination at 30°C in petri plates containing four folds of Whatman filter paper soaked with distilled water.

No nutrient was provided except distilled water after every 24 hrs.
Starch phosphorylase activity was determined during germination of millet seeds starting from zero day up to 7 days.

It was found that activity of the enzyme decreased with germination time, maximum being at the zero day.

Therefore, zero day germinated seeds were used for extraction and partial purification of the enzyme.

From the zero day (the day seeds were soaked), the enzyme activity was determined every day and up to seventh day and a germination profile was drawn as follows:
Fig. 1: Enzyme activity profile of starch phosphorylase in germinating millet seeds
ENZYME EXTRACTION AND PARTIAL PURIFICATION

- All the procedure was carried out at 0-4°C in a cold room.

- The isolation medium → 50 mM tris-HCl buffer, pH 7.6 containing 10 mM 2-mercaptoethanol, 20 mM EDTA and 1% (v/v) triton-X-100.

- Initial Extract → Supernatant containing the enzyme activity.

- Buffer A → 20 mM tris-HCl, pH 7.6 containing 5 mM 2-mercaptoethanol and 20 mM EDTA

- 30-65% ammonium sulfate fraction contained about 72% of the enzyme activity compared to crude extract.
Germinating seeds at various days were blended with 90 ml of isolation medium using a Waring blender for 30 sec at low speed and 60 sec at high speed.

The homogenate was filtered through two layers of a muslin cloth, made the volume 100 ml with the isolation medium and centrifuged at 15,000 x g for 30 min.

After desalting by using Sephadex G-25 chromatography, almost 100% of the enzyme was recovered containing 47 units and 58 mg protein exhibiting specific activity of 0.81 units/mg protein.
To the initial extract, powdered ammonium sulfate was slowly added with constant stirring to get 0-30% saturation.

After storage for 5 hrs, it was centrifuged at 15000 x $g$ for 20 min and the supernatant having most of the activity was brought to 65% saturation with powdered ammonium sulfate.

pH was maintained at 7.6 by the addition of dilute ammonia.

After overnight incubation, the suspension was centrifuged at 15000 x $g$ for 20 min.
The pellet was dissolved in buffer A & centrifuged

The supernatant was desalted using Sephadex-G-25 column chromatography.

The desalted enzyme was used for further study.
The purification profile of the enzyme is shown in following Table.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Enzyme activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>%Recovery</th>
<th>Fold enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>66</td>
<td>254</td>
<td>0.260</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>30-65% fraction</td>
<td>48</td>
<td>59</td>
<td>0.814</td>
<td>72.7</td>
<td>3.13</td>
</tr>
<tr>
<td>Sephadex G-25 Chromatography</td>
<td>47</td>
<td>58</td>
<td>0.810</td>
<td>71.2</td>
<td>3.11</td>
</tr>
</tbody>
</table>
**ENZYME ASSAY**

**In the direction of polysaccharide synthesis**

- The enzyme assay system for the soluble enzyme was consisted of 0.2 ml of 0.2 M tris-maleate buffer, pH 6.0; 0.1 ml of sodium fluoride; 0.1 ml of 3% soluble starch and 0.5 ml of the enzyme preparation and water, pre-incubated at 37°C for 2 min.

  The reaction was started by the addition of 0.1 ml of 0.05 M glucose-1-phosphate.

- After 30 min, the reaction was stopped by the addition of 1 ml of 10% TCA and the tubes were put in an ice bath.

- The precipitate formed was removed by centrifugation in the cold.

- In the clear supernatant, inorganic phosphate formed was estimated using colorimetric method of Fiske and Subbarow (1925).

- The activity of immobilized enzyme was measured using a water bath shaker.
- The assay system was scaled up five times.

- The reaction was stopped by centrifugation at 10000 x g for 5 min in cold condition.

- The amount of the inorganic phosphate formed in the supernatant was measured using the colorimetric method of Fiske and Subbarow (1925).

- One unit of the enzyme activity was taken as the amount of the enzyme required to liberate one micromole of inorganic phosphate in one min under the conditions of the enzyme assay.

**In the direction of glucose-1-phosphate synthesis**

- The enzyme assay system for the soluble enzyme consisted of 0.2 ml of tris-maleate buffer (0.2 M, pH 7.0), 0.1 ml of 5% freshly prepared soluble starch, 0.1 ml of 0.2 M sodium fluoride and 0.5 ml of the enzyme preparation and water, pre-incubated at 37°C for 2 min.
The reaction was started by the addition of 0.1 ml of 0.5 M NaH$_2$PO$_4$-Na$_2$HPO$_4$, pH 7.0.

After 30 min, the reaction was stopped by keeping the tubes in a boiling water bath for 2 min and the precipitate formed was removed by centrifugation in the cold.

In the clear supernatant, glucose-1-phosphate formed was estimated using the phosphoglucomutase and glucose-6-phosphate coupled enzyme assay method (Bergmeyer & Klotzsch, 1965).

The activity of immobilized enzyme was measured using a water bath incubator shaker.

The assay system was scaled up five times.

After one hr, the reaction was stopped by centrifugation at 3000 x $g$ for 10 min in the cold.

The amount of glucose-1-phosphate formed was estimated in the supernatant.
One unit of the enzyme was taken as the amount of the enzyme required to synthesize one micromole of glucose-1-phosphate in one min under the conditions of the enzyme assay.

**PROTEIN ESTIMATION**

- Protein was estimated using the method of [Lowry et al. (1951)](https://doi.org/10.1016/0021-9290(51)90018-5) as modified by [Khanna et al. (1969)](https://doi.org/10.1016/0006-2952(69)90207-2) using bovine serum albumin as a standard.

- In the case of immobilized enzyme, protein was estimated by subtracting the amount of protein present in the concentrated pooled washings of the immobilized enzyme from the amount of the protein taken for immobilization.
ENZYME IMMOBILIZATION

- In the present study, **brick dust** of 1 to 1.5 mm mesh size was washed under running tap water.

- Thereafter, it was treated with 0.5 N hydrochloric acid for 30 min and subsequently washed with distilled water to remove hydrochloric acid.

  This treatment of hydrochloric acid was repeated once.

- After that, washed brick dust was equilibrated with 0.5 M tris-maleate buffer, pH 6.0 (buffer B).

- To 8 ml of desalted enzyme preparation (58 mg protein), 5 gm of equilibrated wet brick dust was added with slow but constant stirring.

- Thereafter, glutaraldehyde was added to the suspension to a final concentration of 0.9% and the suspension was incubated at 0 to 4°C for 4 hrs with frequent but gentle stirring.
After 4 hrs, decantation was done and brick dust was washed with buffer B till no protein was detected in the washings.

Finally, brick dust was suspended in 8 ml of buffer B.

The partially purified enzyme was then subjected to immobilization on brick dust.

The specific activity of the immobilized enzyme was 2.89 units/mg protein whereas the soluble enzyme had 0.81 units/mg protein, thereby immobilized enzyme showed about 3.5 fold purification.

The percentage retention of starch phosphorylase upon immobilization was nearly 80%.

Earlier, Venkaiah and Kumar (1994) showed retention of 46% activity of starch phosphorylase from sorghum leaves using egg shell as solid support.
- **Srivastava et al. (1996)** reported retention of about 50% activity of starch phosphorylase from *Cuscuta reflexa* using egg shell as solid support.

- **Upadhye and Kumar (1996)** reported 79 and 36% retention of starch phosphorylase activity after immobilization on DEAE-cellulose and alginate beads, respectively.
### Immobilization of starch phosphorylase on brick dust

<table>
<thead>
<tr>
<th>Support</th>
<th>Soluble enzyme</th>
<th>Immobilized enzyme</th>
<th>Retention of enzyme activity</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity (units)</td>
<td>Total protein (mg)</td>
<td>Sp. act. (units/mg protein)</td>
<td>Total activity (units)</td>
</tr>
<tr>
<td>Brick dust</td>
<td>47.6</td>
<td>58.3</td>
<td>0.816</td>
<td>38.1</td>
</tr>
</tbody>
</table>
**OPTIMUM pH**

**In the direction of polysaccharide synthesis**

- Enzyme assay was carried out in the range varying from pH 4.5 to pH 8 using tris-maleate buffer to find out the optimum pH at which the enzyme shows maximum activity.

- The soluble enzyme showed optimum pH at pH 6.0 and half-maximal activity at pH 5.4 and 7.0 whereas immobilized enzyme showed optimum pH at 6.2 and half maximal activity at pH 5.7 and 7.0.

**In the direction of glucose-1-phosphate formation**

- Enzyme assay was carried out in the range varying from pH 6.2 to pH 8.5.
The soluble enzyme showed optimum pH at pH 7.6 and half maximal activity at pH 6.7 and 7.9.

After immobilization, the enzyme showed maximal activity at pH 7.7 with half maximal activity at 6.8 and 8.0.
OPTIMUM pH

In the direction of Polysaccharide synthesis

Enzyme showed Optimum activity At pH 6.0

Two half pH optima at pH 5.4 & pH 7.0

Enzyme showed Optimum activity at pH 7.6

In the direction of Glucose-1-phosphate formation

Two half pH Optima at pH 6.7 & pH 7.9
pH- enzyme activity (in the direction of polysaccharide synthesis) relationship of starch phosphorylase

![Graph showing pH-enzyme activity relationship](image)
pH- enzyme activity (in the direction of polysaccharide degradation) relationship of starch phosphorylase
OPTIMUM TEMPERATURE

In both polysaccharide synthesis and glucose-1-phosphate formation directions

- Enzyme assay was carried out in the range of 20°C to 55°C to find out the optimum temperature.

- The soluble enzyme showed optimum temperature at 37°C and the half-maximal activity at 34°C and 40°C whereas immobilized enzyme showed optimum temperature at 45°C and half maximal activity at 38 and 50°C
Consistent with the present results, Makkar and Sharma (1983) in the case of *Lactobacillus bulgaricus* β-galactosidase, and our own laboratory in cases of potato starch phosphorylase (Venkaiah & Kumar, 1995), *Cuscuta reflexa* starch phosphorylase (Srivastava *et al.*, 1996) cabbage leaves starch phosphorylase (Garg & Kumar, 2007) also reported a considerable increase in the optimum temperature upon immobilization on egg shell.

However, in our laboratory itself, in the case of Bengal gram starch phosphorylase, decrease in the optimum temperature was noted upon immobilization both on DEAE cellulose and alginate beads (Upadhye & Kumar, 1996).
OPTIMUM TEMPERATURE

In the direction of Polysaccharide synthesis:
- Enzyme showed Optimum temp. of 37 degree Celsius
- Two half optima temp at 34 & 40 degree Celsius

In the direction of Glucose-1-phosphate formation:
- Enzyme showed Optimum temp. of 37 degree Celsius
- Two half optima temp at 34 & 40 degree Celsius
Temperature-enzyme activity (in the direction of polysaccharide synthesis) relationship of starch phosphorylase.
Temperature-enzyme activity (in the direction of poly-saccharide degradation) relationship of starch phosphorylase.
**THERMAL STABILITY**

- Thermal stability studies were carried out by incubating the enzyme in capped tubes in a water bath at different temperatures between 30 and 55°C.

- Every 30 min, suitable aliquots were withdrawn from the tubes and stored on ice before enzyme assay.

- The soluble enzyme had a half life of 5 hr at 30°C and 30 min at 50°C.

- Immobilized enzyme had a longer half life of 9 hr at 30°C and 1 hr at 50°C.

- The increased thermal stability of the immobilized enzyme is very useful for the continuous production of glucose-1-phosphate from starch.

- Our present results are consistent with our earlier results on immobilization of sorghum leaves, *Cuscuta reflexa* and cabbage starch phosphorylases (Venkaiah & Kumar, 1995; Srivastava *et al.*, 1996; Garg & Kumar, 2007).
ENZYME REUSABILITY

- Each day, starch and sodium phosphate buffer were passed through the immobilized enzyme column at 30°C for 5 hrs followed by washing with 5 times the bed volume of 0.02 M tris-HCl buffer, pH 7.5 and incubation under cold conditions.

- A single fraction was collected on each run and glucose-1-phosphate in the effluent was determined by the coupled enzyme assay method (Bergmeyer & Klotzsch, 1965).

- The 20 batches could be carried out with almost same efficiency and thereafter efficiency decreased.

- The property of reuse up to so many times may be exploited in the production of glucose-1-phosphate.

- The effluent collected from the immobilized enzyme column contained small amounts of starch and inorganic phosphate as contaminants with glucose-1-phosphate.
The contaminants were found to be removed by adsorption chromatography through a norite-celite (2 :1) column (1.5 x 10 cm), previously equilibrated with distilled water, introduced as a glucose-1-phosphate trap with the column.

Glucose-1-phosphate was eluted from the column at a flow rate of 10 ml/hr using water as the eluent.

The effluent was further purified by using ion-exchange chromatography using a DEAE cellulose column previously equilibrated with distilled water.

Glucose-1-phosphate was eluted from the column using 0.02 M potassium acetate buffer, pH 7.0.

The product thus obtained was precipitated with two volumes of 95% ethanol and dried in a vacuum oven at 60°C.

The final product was tested using coupled enzyme assay and found to be nearly 95% pure.
GLUCOSE-1-PHOSPHATE (PRODUCT) PURIFICATION

- The effluent collected from the immobilized enzyme column was passed through a norite-celite (2:1) column (1.5 x 10 cm), previously equilibrated with distilled water, introduced as a glucose-1-phosphate trap with the column.

- Glucose-1-phosphate was eluted from the column at a flow rate of 10 ml/h using water as the eluent.

- The effluent was further purified by ion-exchange chromatography using a DEAE cellulose column (1.5 x 10 cm) previously equilibrated with distilled water.
  - Glucose-1-phosphate was eluted from the column using 0.02 M potassium acetate buffer, pH 7.0 at a flow rate of 10-12 ml/hr.
  - The product thus obtained was precipitated with two volumes of 95% ethanol and dried in a vacuum oven at 60°C.
The final product was tested using phosphoglucomutase and glucose-6-phosphate dehydrogenase coupled enzyme assay (Bergmeyer and Klotzsch, 1965).
ACKNOWLEDGEMENTS

We thank Avinash Kumar Singh for his assistance in the present work. The authors acknowledge the facilities of the Department of Biotechnology, Government of India, New Delhi (DBT) under M.Sc. Biotechnology program. Facilities of DBT’s Bioinformatics Sub Center are also gratefully acknowledged.