CHAPTER FOUR

Immobilization of Laccase

4.1 Introduction

The immobilization of enzymes has been accomplished by several procedures, including covalent attachment to various matrixes, adsorption to insoluble substances, or entrapment within a matrix. Such insoluble preparations have been useful not only for industrial applications but also in the study of multienzyme systems and as tools in biological research (Cuatrecasas and Arfinsen, 1971; Mosbach, 1971, and Hartmeier, 1988). The choice of the support is determined by the conditions under which the enzyme is to be immobilised and by the coupling method involved.

In this study, the immobilization of laccase by entrapment in copper alginate gel was studied. The success of this technique was due mainly to the gentle environment it provides for the entrapped material.

4.2 Materials and Methods

4.2.1 Materials

Insoluble matrixes. Alginic acid from *Macrocytis pyrifera*, of low viscosity was purchased from Sigma, Chemical Co. St. Louis, MO.

Enzyme. Crude enzyme was obtained from Novo Nordisk, Malaysia.
4.2.2 Immobilization Method

Preparation of immobilized laccase with copper alginate gel

The enzyme was immobilized by entrapment in copper alginate beads. A 100μl bovine serum albumin (BSA) solution, containing 160U laccase was mixed with 10ml 3% (w/v) sodium alginate solution. The resulting homogenous mixture was centrifuged at 4,000 revolutions per minute for five minutes to remove air bubbles, and extruded drop by drop using a syringe attached to a 21 gauge needle into 0.15 M aqueous CuSO₄ solution at pH 4.0. The beads were left in the solution and stirred overnight.

The spherical blue beads were then washed exhaustively with distilled water until pH of the wash water reached pH 5.0-5.5. The diameter of the beads was in the range of 1.5 to 2.5 mm, and the total wet weight of beads obtained from 10ml of sodium alginate solution was about 7g. The enzymatic activity entrapped in the formed beads was determined by measuring the laccase activity.

4.2.3 Optimization of Immobilization

Effect of pH

The pH of the immobilization buffer was varied from 3.5 to 9.0

Effect of laccase concentration on immobilization

Different amounts of crude laccase ranging from 1 mL to 6 mL were added to the
immobilization solution. The immobilization buffer for copper alginate was 0.05M phosphate buffer at pH 6.0.

4.2.4 Activity of free and immobilized enzyme

_Determination of optimum pH_

The assays for free and immobilised laccase activities for entrapment method were carried out. The pH of the assay buffer was varied from 3.7 to 9.0.

_Determination of optimum temperature_

The activity of free and immobilized laccase was assayed at 4°C, 20°C, 30°C, 40°C, 50°C and 60°C.

4.2.5 Stability studies of free and immobilized enzyme

_Determination of thermal stability_

Thermal stability of the free and immobilized laccase was evaluated by measuring the residual activity of laccase exposed to various temperatures in 0.05M phosphate buffer at optimum pH for 15 minutes. After exposing, they were quickly cooled to 25°C and assayed for enzymatic activity at that temperature. The remaining activities were expressed as relative to the original activities assayed at 25°C without heating.
Storage Stability

The immobilized laccase by entrapment method was stored in 0.05 phosphate buffer of pH 6.0, at 4°C and 25°C for 15 days. Its activity was measured at specific intervals. The immobilized laccase by entrapment method was stored in 0.05M sodium citrate buffer of pH 4.0 and kept at 4°C and 25°C for 15 days. Its activity was measured at specific intervals.

4.2.6 Analytical Techniques

Preparation of substrate to estimate laccase activity

0.1mM syringaldazine was dissolved in 50% ethanol (Harkin and Obst, 1973). It takes about 13h to dissolve 0.1mM syringaldazine completely in 50% ethanol with constant stirring.

Estimation of soluble laccase activity

The activity of laccase was assayed at 30°C in 3.0 ml of 0.05M phosphate buffer of pH 6.8 containing 0.1 ml enzyme and 0.1ml of 0.1mM syringaldazine (in ethanol) as substrate (Harkin and Obst, 1973). One unit of enzyme activity is defined as the amount of enzyme producing an OD change of 1 unit/min/g substrate at 527 nm.
Estimation of immobilized laccase activity entrapped in copper alginate

Ten beads of laccase entrapped in copper alginate were incubated in 3.0 ml 0.05M phosphate buffer pH 6.0 containing 0.1ml of 0.1mM syringaldazine in ethanol. The mixture was incubated at 30°C for 30 minutes in a horizontal shaker. One unit of enzyme activity is defined as the amount of enzyme producing an OD change of 1 unit unit/min/g substrate at 527 nm.
RESULTS AND DISCUSSION

Influence of pH on immobilization of the laccase is shown in Fig. 4.1. The optimum pH of the immobilized laccase obtained by the copper alginate method was found to be 4.0 with a rather sharp peak. This effect is in agreement with a general observation (Miyama et al., 1985; Kobayashi et al., 1992) that positively charged supports displace pH activity curves of enzymes attached to them towards lower pH values. When immobilization was performed at pH values higher than 4.0, a decrease in activity was observed. This might be attributed to a modification of the enzyme structure which lowers catalytic activity.

Optimum pH for activity of free and immobilized laccase

The optimum pH for activity of the free commercial enzyme is pH 5.5 in 0.05M phosphate buffer (Fig. 4.2). The % activity for both the free and immobilized enzyme increased exponentially until it reached a maximum value and decreased exponentially and achieved its original activity of about 10% activity at pH 6.0. However, the optimum pH of activity for the immobilized enzyme was shifted towards the acidic side by 0.5 units for the the entrapped enzyme (pH 5.0). Similar results were observed for pepsin immobilized on sand which shifted the pH optimum by 0.3 units (Puvanakrishnan et al., 1984). A maximum displacement of 2 units from the optimum pH of native enzyme was observed for trypsin immobilized on sand via glutaraldehyde.

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Fig 4.1: Effect of pH of immobilisation of laccase in copper alginate
Fig 4.2: Effect of pH on the activity of free and immobilized laccase

- Free enzyme
- Immobilized enzyme
(Puvanakrishnan et al., 1980). The shift in pH optimum might be due to chemical modification of the enzyme and the net charge of the support material (Erarslan and Guray, 1990). Laccase from *Coriolus versicolor* immobilized on activated carbon maintained greater activity at lower pH values than soluble laccase (Davis and Burns, 1992).

**Effect of enzyme concentration on immobilization**

When the amount of laccase was increased during immobilization, the activity of the immobilized enzyme was found to increase (Fig. 4.3). Lacasse of 300 µl (480 U/150 alginate beads) was found to provide the maximum level of activity of immobilized laccase. Above this amount a reduction in the activity was observed. Similar observations were made by Chellapandian and Sastry, (1994) with alkaline protease immobilized on nylon. The decrease in activity for higher concentrations of the enzyme may be due to (a) enzyme-enzyme interactions at a saturated state thus limiting its capacity and (b) stearic hindrance. Activity of fungal laccase immobilized on porous glass beads increased linearly and the reactive groups appear to be saturated above 20 U/mg glass beads (Leonowicz *et al.*, 1988).
Fig 4.3: Effect of enzyme amount (concentration) on immobilization of laccase
Effect of temperature on activity

The optimum temperature of free and immobilized laccase activity using the entrapment method was found to be at 30°C. The optimum temperature for immobilized laccase activity increased with temperature up to a certain point after which, due to the denaturation of the enzyme, a decline in the activity was observed (Fig. 4.4). At 60°C the free enzyme lost about 50% of its original activity. The entrapped enzyme showed about 50% lower activity at 60°C with respect to the original activity. However, the activity of the entrapped enzyme was higher than the free enzyme by 10% at 4°C and 5% higher at 60°C. Studies reported by Gianna Palmieri et al. (1994) showed that the optimum temperature for the activity of the immobilized phenol oxidase shifted to lower temperature values with respect to that of the free form. Laccase immobilized on activated carbon displayed optimum temperature at 40°C. In this study both the free and immobilized enzyme had a 50% reduction in activity at 60°C. The activity of the immobilized enzyme was only 5% higher than the free enzyme at 4°C. The objective of immobilization is the economic application of enzyme systems. Therefore, considering the cost of immobilization it is not advisable to work at temperatures above 30°C.
Fig 4.4: Optimum temperature activity of free and immobilized laccase in copper alginate

![Graph showing temperature activity of free and immobilized laccase in copper alginate. The graph displays two curves: one for free enzyme (■) and another for laccase-alginate (○). The x-axis represents temperature in °Celsius, and the y-axis represents % Activity.]
Thermal stability of free and immobilized enzyme

The effect of temperature on the stability of laccase entrapped in copper alginate and free enzyme is shown in Figure 4.5. The free and immobilized enzyme were almost 100% stable from 4°C to 30°C after which there was a steep drop in stability. The activity of free enzyme was 15% at 60°C while that of immobilized laccase was 20% at the same temperature.

Studies carried out by Leonowicz et al. (1988) showed that laccase from Trametes versicolor immobilized onto porous glass beads and the free enzyme was thermally stable up to 40°C followed by a steep drop in stability for the free enzyme. The stability in the immobilized laccase gradually declined with increase in temperature. Enzyme immobilized on glass showed in general increased thermal stability compared to organic carriers (Weetall, 1970).

Surprisingly, the immobilized enzyme system of manganese peroxidase from Phlebia radiata and glucose oxidase from Aspergillus niger when immobilized on porous silica beads (Aken et al., 2000) showed a lower thermal stability than the free enzyme. Immobilized enzymes are generally more stable against thermal denaturation than the free enzyme. However, improvement by immobilization is not a general rule.
Fig 4.5: Thermal stability of free enzyme and laccase in copper alginate (pH 5.5)
Storage Stability

The effect of storage time on the activity of the free and immobilized enzyme was also studied. Storage at 25°C saw a sharp drop in activity in both the free and immobilized enzyme. Approximately about 50% -55 % activity was retained after two days of incubation. The drop in activity was linear up to the next eight days. The drop in activity from the tenth to the fourteenth day was not very significant as it was below 5% activity. The same profile was seen for storage at 4°C. The apparent half-life at 4°C for both the free and immobilized enzyme was around five days. The drop in activity was linear for the next nine days. Laccase immobilized on activated charcoal during storage in distilled water at 4°C lost about 38% activity after four days of storage (Davis and Burns, 1992). Co- immobilization of manganese peroxidase and glucose oxidase was more stable than the free enzyme by 24% after six weeks of storage at 4°C (Aken et al., 2000).
Fig 4.6: Storage stability at 25°C
Fig 4.7: Storage Stability at 4°C

![Graph showing storage stability at 4°C with two lines representing free enzyme and immobilized enzyme.](image)