# CHAPTER FOUR

## 4.0 RESULTS AND DISCUSSION

## 4.1 IMMOBILIZATION OF UREASE ON NYLON 6

### 4.1.1 Studies on immobilization parameters

Nylon-6 was partially hydrolyzed with hydrochloric acid to release carboxyl and amino groups present in it. The amino group of Nylon-6 was activated with glutaraldehyde to yield carbonyl derivatives and then treated with the enzymes. Immobilization of the enzymes is based on the formation of a covalent bond between the enzyme molecules and Nylon-6. The reaction involved is Schiff's base formation. The aldehyde derivaties of the Nylon-6 reacts with the amino groups of the enzymes and forms a Schiff's base.

The immobilization parameters such as optimum immobilization pH, reaction time and amount of enzyme were studied.

Influence of pH on immobilization of the urease on Nylon-6 tube is shown in Figure 4.1. The optimum pH of immobilized urease obtained by the nylon-glutaraldehyde-urease conjugate was found to be 6.0. Similar results were obtained (Jiugao *et al.*, 1994) with urease immobilized on periodate oxidised starch. As shown in Figure 4.1, a small shift from the optimum pH was found to

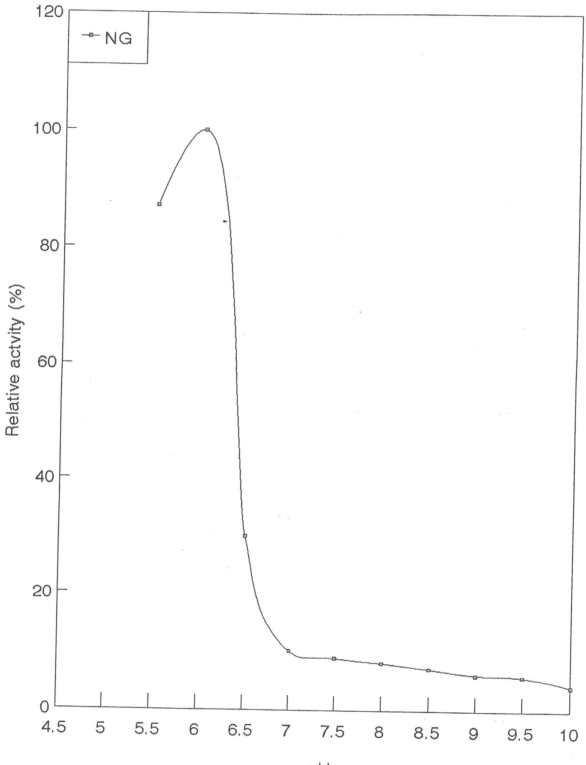




Fig 4.1. Effect of immobilization pH on the activity of urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

cause significant reduction in the relative activity of the immobilized urease. 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 the amount of urease was increased during immobilization, the relative activity of the immobilized enzyme was found to increase (Figure 4.2). Urease concentrations of 7 mg/ml was found to provide the maximum level of activity of immobilized urease. Above these concentrations for immobilized urease, a reduction in the relative activity was observed. Similar observations were made by Chellapandian and Sastry, (1994) with alkaline protease immobilized on nylon. The decrease in relative activity for higher concentrations of the enzyme may be due to enzyme-enzyme interactions and stearic hindrance.

As shown in Figure 4.3, nylon tubes used were found to saturate within 3 hours of incubation at 4°C, beyond which there was no further increase in the relative activity. Tarafdar and Chhonkar, (1982) found that when urease was quickly adsorbed on clay surfaces, equilibrium was attained within one hour of incubation. Urease has a high molecular weight and its rapid adsorption can be attributed to this characteristic. However, Jiugao *et al.*, (1994) found that immobilization of urease on starch was most effective when the reaction time was 24 hours.

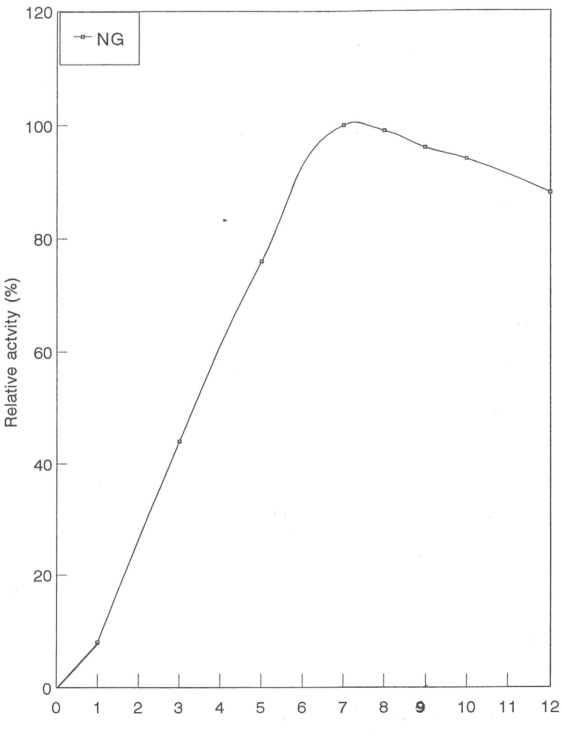


Fig 4.2. Effect of enzyme amount on the activity of urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

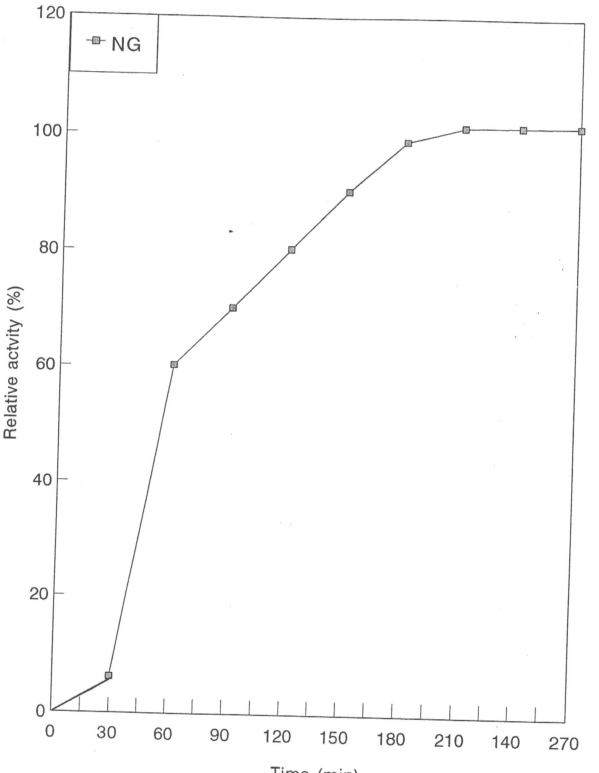


Fig 4.3. Effect of incubation period on the activity of urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

The maximum activity of immobilized urease on Nylon-6 tubes was obtained at immobilization pH of 6.0, 7 mg/ml of urease and incubation time of 3 hours at $4^0$ C. At these conditions, 0.635 mg of urease per 100 mg of Nylon-6 was found to get coupled. The specific activity retained was 12.11% of free urease. The specific activity of free urease was 31.947 Mm/min/mg protein.

### 4.1.2 Properties of soluble and immobilized urease

The effect of temperature on the reaction rate are as shown in Figure 4.4, the highest relative activity of immobilized urease was obtained at 65°C. Activity of free urease was found to be maximum at 25°C. The significant increase in the optimum temperature when urease was bound to nylon indicated that immobilized urease resisted denaturation due to temperature rise. Similar observation was made by Iyengar and Rao, (1979) using urease bound to chitin and by Lai and Tabatabai, (1992). Results obtained by Pozniak *et al.*, (1995) using urease bound to modified polysulphone membrane showed that the free urease (Type III) had an optimum temperature of about $62^0$ C, whereas the optimum temperature of the immobilized urease shifted to $70^0$ C. The increased adsorption of protein with increasing temperature was reported to be due to the unfolding of the protein molecules, having more functional groups available for binding to the support (Weetall and Messing, 1972). The optimum temperature for immobilized urease activity increased with the increase in temperature up to a certain point after which, due to the denaturation of the enzyme, a decline in the activity was observed (Figure 4.4).

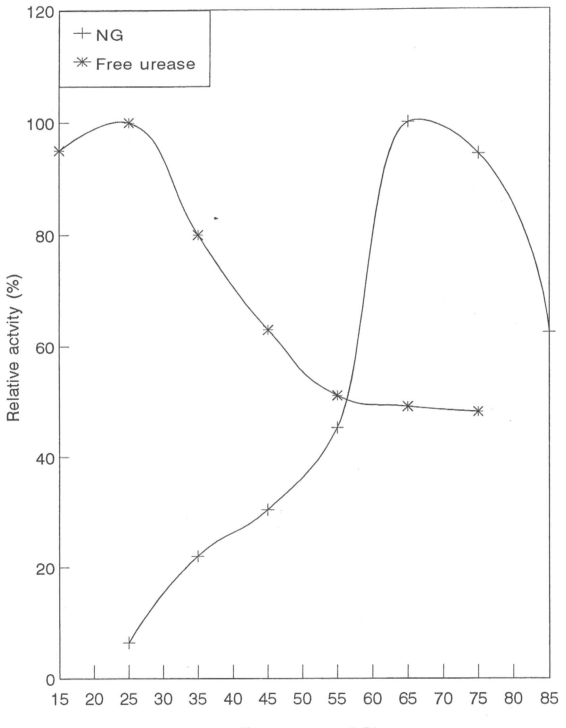


Fig 4.4. Effect of temperature on the activity of free urease and urease immobilized on nylon tube (NG). The enzyme assay was carried out various temperatures for 15 minutes at buffer pH of 7.0.

The effect of temperature on the stability of urease immobilized on partially hydrolysed nylon tubes and free urease is shown In Figure 4.5. The activity of free enzyme retained was found to be 30% at 60°C while that of immobilized urease was 61% at the same temperature. The immobilized urease was found to have higher thermal stability when compared to the free enzyme. The improved thermal stabilify of the immobilized urease is mainly due to multipoint attachment of enzyme to support. Results reported by Gianfreda *et al.*, (1992) showed that the immobilized urease had a higher sensitivity to temperature than the free enzyme. These results agree with those reported by Boyd and Mortland, (1985) for urease bound on HDTA-smectite and Pozniak *et al.*, (1995) but are in contrast to the findings of Sundaram and Crook, (1971). The latter found that jackbean urease adsorbed on kaolinite was as thermally stable as the free enzyme Krajewsjka *et al.*, (1990); Yoshiki and Yasumi, (1980) found that immobilized urease had a higher thermal stability when compared to the free urease.

The pH effect on the activity of urease in immobilized and free state is shown in Figure 4.6. Immobilization did not shift or alter the optimum pH of the enzyme. Similar observations were made by Sundaram and Crook, (1971); Tarafdar, (1982); Lai and Tabatabai,(1992) and Boyd and Mortland, (1985). As illustrated in Figure 4.6, the optimum pH for both the immobilized urease and free urease was 6.5. It was reported that urease had different pH optima ranging from

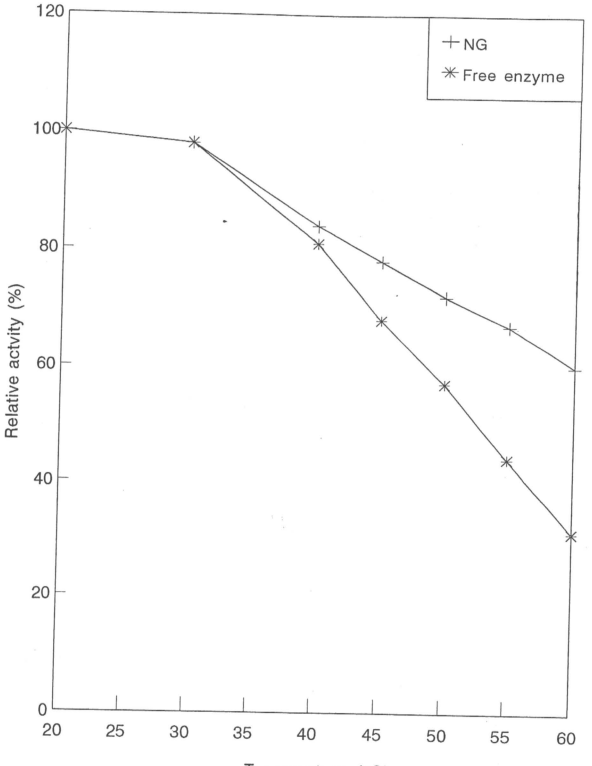


Fig 4.5. Effect of temperature on the stability of free urease and urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

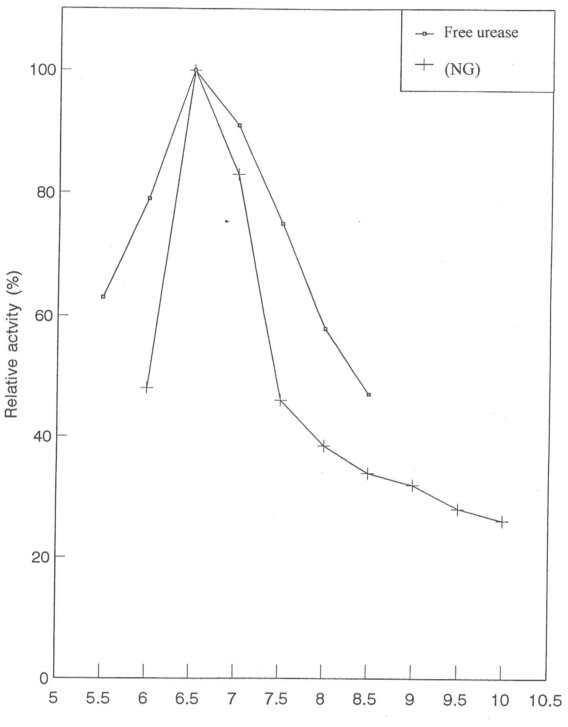


Fig 4.6. Effect of buffer pH on the activity of free urease and urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C. The buffer pH was varied.

6.4 to 7.4 depending on the type of buffer used (Rehn and Reedm 1987). As reported by Pozniak *et al.*, (1985), Iyengar and Rao, (1979) and Weetall and Hersh, (1969) when bound to a support, the optimum pH of urease shifted towards the more acidic side. However, when urease was activated with formaldehyde (Krysteva *et al.*, 1991), the pH optimum shifted towards the alkaline side.

Figure 4.7 shows that immobilized urease retained 76% and 51.7% of its original activity after 60 days when stored at 4°C and at 25°C respectively.

Maximum activity of immobilized urease and free urease were obtained at a substrate urea concentration of 4%. The reusability of the immobilized urease for urea hydrolysis is important. Table 4.1 illustrates the effect of repeated use of immobilized urease on the residual activity of urea hydrolysis. The immobilized urease retained 78% of its original activity after 5 repeated uses. However, results by Pozniak *et al.* (1995) showed that after 17 reuses, the immobilized urease retained 60% of its initial activity.

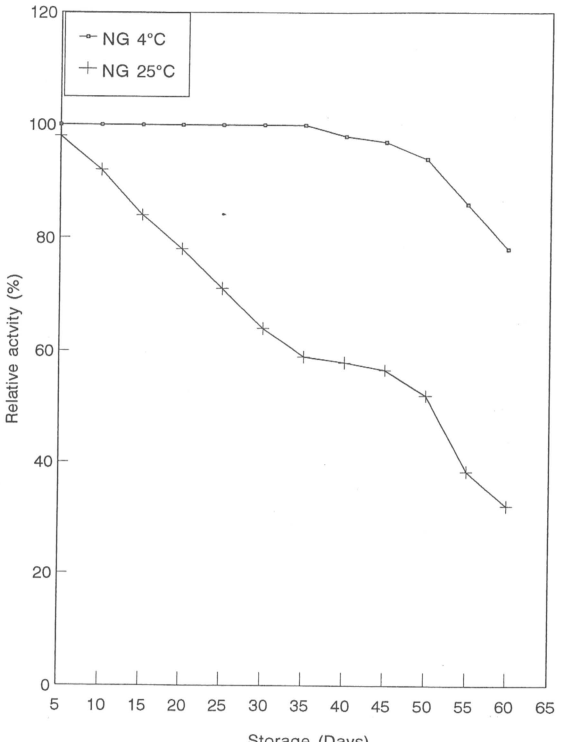


Fig 4.7. Storage stability of urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

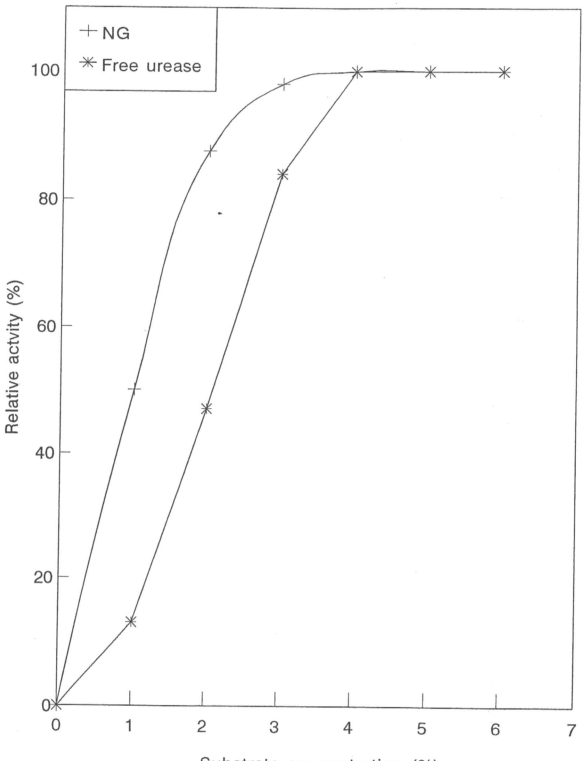


Fig 4.8. Effect of substrate concentration on the activity of free urease and urease immobilized on nylon tube (NG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

**Table 4.1 : Reusability of immobilized urease on Nylon-6 tube**

| Number of times used | Retained activity (%) |
|---|---|
| 1 | 95 |
| 2 | 90 |
| 3 | 87 |
| 4 | 83 |
| 5 | 78 |

## 4.2 ADSORBTION OF UREASE ON VERMICULITE SATURATED WITH CATIONS

The cations with which the vermiculite were saturated influenced significantly the adsorption of urease. The aluminium saturated vermiculite adsorbed maximum amount of enzyme followed by calcium and sodium saturated ones. It has been reported that the activity of soyabean urease immobilized on cationic vermiculite increased in the order of $Na^{+}$, $Ca^{2+}$ and $Al^{3}$ (Tarafdar, 1987). The lesser amount of adsorption of enzyme observed on potassium saturated vermiculite may be due to the fixation of patassium on vermiculite, whereas the higher amount of enzyme adsorbed on the aluminium and calcium saturated vermiculite is due to ion exchange property (Ensminger and Giesking, (1941).

The increase of temperature increased the adsorption of urease in all samples as seen in Figure 4.9. Tarafdar (1987) reported that the increase of temperature increased the adsorption of urease. The increased adsorption of protein with increasing temperature may be due to the unfolding of the protein molecules, having more functional groups available for binding to the support (Weetall and Messing, 1972).

As shown in Table 4.2, equilibrium was attained within three hours of incubation at 45°C. The amount of enzyme adsorbed was in the increasing order when potasium, calcium and aluminium saturated vermiculite are used. This

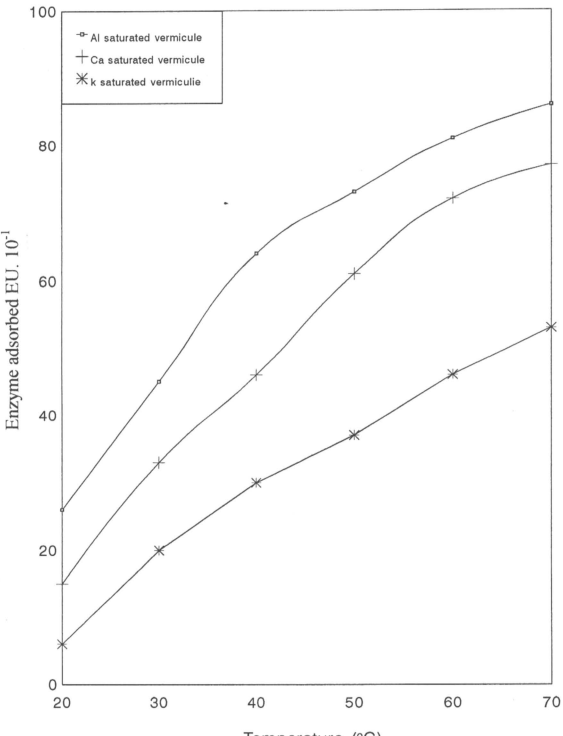


Fig 4.9: Effect of temperature on adsorption of urease immobilized on cationic vermiculite

shows that aluminium, saturated vermiculite adsorbed more enzyme in three hours of incubation. This may be due to high molecular weight of enzyme and rapid adsorption.

**Table 4.2:** Effect of time on adsorption of urease on vermiculite saturated with different cations

| Time | Enzyme adsorbed EU x $10^{-1}$ per 100 mg | | |
|---|---|---|---|
| (min) | Aluminium saturated | Calcium saturated | Potassium saturated |
| 30 | 62.1 | 51.5 | 34.5 |
| 60 | 72.3 | 62.5 | 48.2 |
| 90 | 82.3 | 70.3 | 60.4 |
| 120 | 86.2 | 76.4 | 68.2 |
| 150 | 90.0 | 84.5 | 78.9 |
| 180 | 93.1 | 89.7 | 81.8 |
| 210 | 93.6 | 90.1 | 82.1 |

In all the three cases of vermiculite saturated with cations, 80 mg of vermiculite per ml of enzyme was found to give optimal adsorption of the enzyme as shown in Table 4.3. Beyond this concentration, percent adsorption of enzyme did not increase and this may be attributed to non availability of the enzyme substrate Armstrong and Chesters, 1964. The aluminium saturated vermiculite adsorbed maximum amount of urease compared to calcium and potassium saturated vermiculite. Tarafdar and Chhonkar (1982), reported that with soyabean urease linear relationship between clay concentration and urease adsorption was obtained only up to 40 mg of clay per ml. Beyond this concentration, percent adsorption of enzyme decreased.

**Table 4.3:** Effect of cation saturated vermiculite dose on adsorption of urease

| **Vermiculite** | **Enzyme adsorbed EU x $10^{-1}$ per 100 mg** | | |
|---|---|---|---|
| **dose (mg/ml)** | **Aluminium saturated** | **Calcium saturated** | **Potassium saturated** |
| 10 | 52.5 | 38.6 | 25.8 |
| 20 | 66.2 | 52.3 | 38.2 |
| 40 | 74.1 | 63.7 | 53.9 |
| 60 | 82.3 | 72.1 | 64.5 |
| 70 | 88.6 | 83.4 | 77.2 |
| 80 | 92.8 | 89.1 | 81.3 |
| 100 | 93.0 | 89.6 | 82.1 |

The maximum adsorption of urease on vermiculite saturated with cations occured at pH 6.0 (Figure 4.10). The reduction in the amount of enzyme adsorbed with decrease in pH may be due to competition of hydrogen ions for the adsorption sites on the mineral surface. When pH was raised above isoelectric pH, the negative charge on the protein increased, thereby the protein molecules became less positive and eventually negative. Thus the possibility for the clay or mineral to attract more protein molecules with increasing pH is counteracted by the tendency of the protein to become negatively charged. This may account for lower adsorption of enzyme at very high pH of the vermiculite enzyme system.

The adsorption of enzyme was found to be concentration dependent (Table 4.4). The extent of adsorption by different vermiculte samples increased with increasing concentration of the enzyme up to $300.10^{-1}$ EU per 100 mg vermiculite. The initial rapid adsorption could be attributed to the binding of the enzyme to random sites on vermiculite surface. The adsorption isotherms obtained were nearly linear on log-log plot, suggesting applicability of the Freundlich isothern. The Freundlich equation is expressed as follows (Dean, 1949).

$$X/m = Kc_e^{1/n},$$

where $X/m$ is the quantity adsorbed per unit mass of adsorbent, $C_e$ is the concentration of solute in equilibrium with adsorbent, K and l/n are constants denoting respectively, the intercept at unit concentration and the slope of log-log

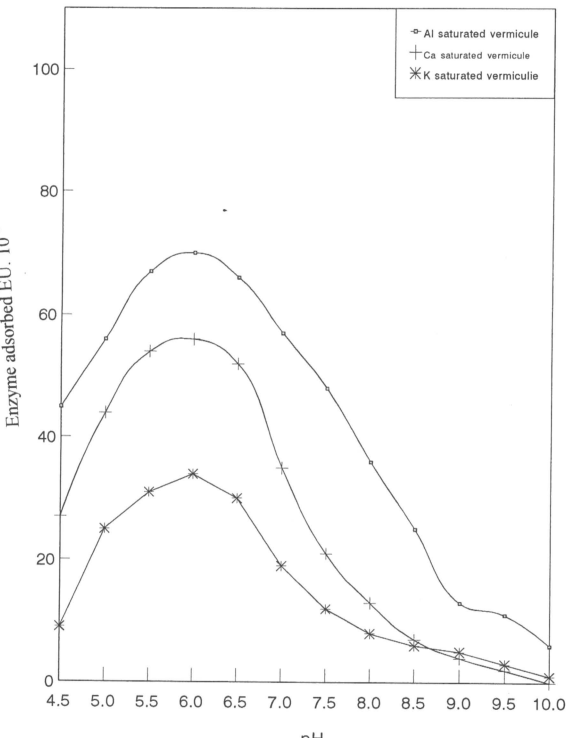


Fig 4.10. Effect of pH on adsorption of urease immobilized on cationic vermiculite

plot of $X/m$ vs $C_e$. K is proportional to the amount of enzyme adsorbed when $C_e$ equals one and provides estimate of adsorbent capacity. The constant n indicates the intensity of adsorption. Both the values of K and n computed from adsorption isotherms of urease in vermiculite showed (Table 4.5) that they were highest in aluminium saturated vermiculite followed by calcium and potassium saturated vermiculite. This is mainly due to exchanged ions where the exchanged ions are probably held more strongly in the increasing order of monovalent, divalent and trivalent ions. Trivalent ions are usually adsorbed so strongly on ion exchange material that they frequently are not removed by washing (Chellapandian and Sastry, 1992).

**Table 4.4:** Effect of enzyme concentration on adsorption

| **Enzyme amount (Eux10 )** | **Enzyme adsorbed EU x $10^{-1}$ per 100 mg** | | |
|---|---|---|---|
| | **Aluminium saturated** | **Calcium saturated** | **Potassium saturated** |
| 50 | 30 | 28 | 27 |
| 100 | 51 | 45 | 43 |
| 150 | 68 | 61 | 56 |
| 200 | 79 | 74 | 69 |
| 250 | 90 | 85 | 76 |
| 300 | 94 | 89 | 80 |

**Table 4.5:** Freundich adsorption parameters of cation saturated vermiculites

| Time | Enzyme adsorbed EU x $10^{-1}$ per 100 mg | | |
|---|---|---|---|
| (min) | **Aluminium saturated** | **Calcium saturated** | **Potassium saturated** |
| k | 2.05 | 1.00 | 1.78 |
| l/n | 0.853 | 0.892 | 1.06 |
| n | 1.172 | 1.121 | 0.943 |

Figure 4.11 shows that immobilized urease retained (i) 69%, (ii) 30%, (iii) 55%, (iv) 19%, (v) 47%, (vi) 10% of its original activity after 60 days when stored as (i) aluminium saturated vermiculite at 4°C, (ii) aluminium saturated vermiculite at 25°C, (iii) calcium saturated vermiculite at 90°C, (iv) calcium saturated vermiculite at 25°C, (v) potassium saturated vermiculite at 4°C, and (vi) potassium saturated vermiculite at 25°C. It was observed that there was loss in activity on storage at 25°C. The loss in activity on storage at 25°C might be due to denaturation of the bound enzyme.

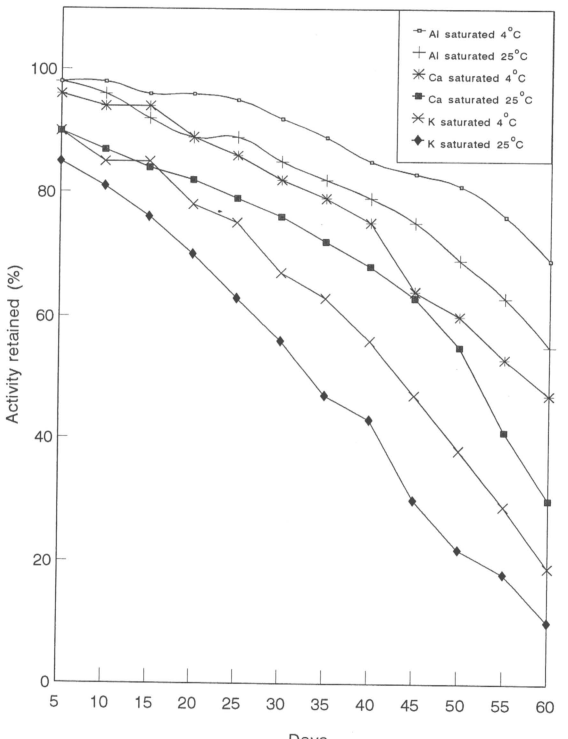


Fig 4.11. Storage stability of urease immobilized on cationic vermiculite

## 4.3 IMMOBILIZATION OF UREASE ON VERMICULITE-GLUTARALDEHYDE

### 4.3.1 Studies on immobilization parameters

Vermiculite was coupled to urease (method 1) and to urease using glutaraldehyde (method 2). The optimum pH for urease immobilized by vermiculite-urease-EDTA (Method 1) was found to be 6.0 with a broad pH range while that for urease immobilized by vermiculite-glutaraldehyde EDTA-urease conjugate (Method 2) was 5.5 with a rather sharp peak. The effect of immobilization pH on immobilization is presented in Figure 4.12. When enzyme coupling was performed at higher pH value, a decrease in activity was observed. This might be attributed to a modification of the enzyme structure on alkaline medium which lowers fixation ability and catalytic activity of the enzyme molecules. Therefore, for further studies a urease solution of pH equal to pH 6.0 (method 1) and pH 5.5 (method 2) respectively was chosen.

By varying the concentration of urease in phosphate buffer of pH 6.0 (method 1) and pH 5.5 (method 2) respectively, it was found that urease concentrations of 10 mg/ml and 8 mg/ml were found to provide the optimum level of activity of immobilized urease obtained by Methods 1 and 2 respectively (Figure 4.13). Above these urease concentrations, for immobilized ureases obtained by Methods 1 and 2, a reduction in the relative activity was observed. Similar observations were made by Chellapandian and Sastry (1994), with alkaline

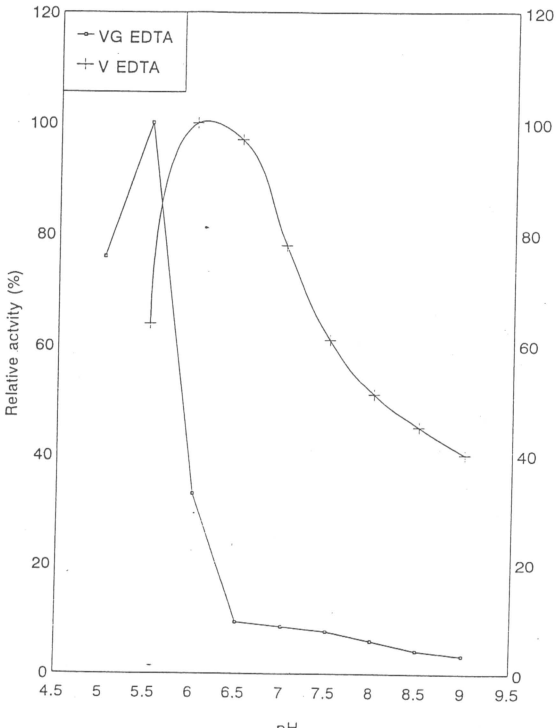


Fig. 4.12. Effect of immobilization pH on the activity of urease immobilized on vermiculite-EDTA (V EDTA) and vermiculite-glutaraldehyde-EDTA (VG EDTA). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

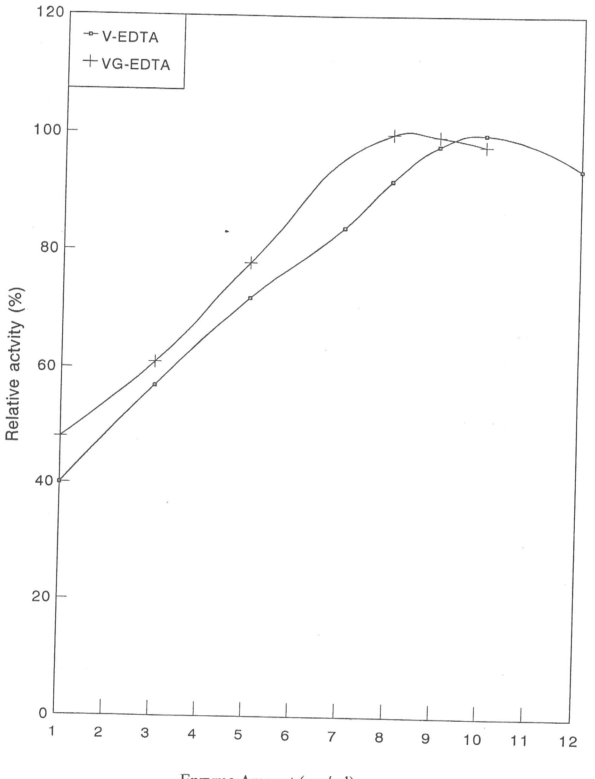


Fig. 4.13. Effect of enzyme amount on the activity of urease immobilized on vermiculite-EDTA (V-EDTA) and vermiculite-glutaraldehyde-EDTA (VG-EDTA). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

protease. To optimize the enzyme immobilization procedure, it is important to determine the capacity of the support for the greatest retention of enzyme activity.

When the urease coupling time was varied from 30 minutes to 210 minutes, the carrier used in both methods was found to reach saturation within 3 hours of incubation (at 4°C). Beyond this, there was no further increase in relative activity. From Figure 4.14, it can be seen that vermiculite adsorbed more enzyme within one hour in Method 1 compared to that adsorbed in Method 2.

Based on these results, a method of urease immobilization on the vermiculite was developed as follows. The vermiculite was immersed in a solution of urease in phosphate buffer of pH 6.0 (method 1) and pH 5.5 (method 2) respectively at a urease concentration of 10 mg/ml for method 1 and 8 mg/ml for method 2 respectively. The mixture was allowed to stand for 3 hours at room temperature(25$^{o}$C). After that time the vermiculite was washed with phoshate buffer until washings were free of urease. At these conditions, the amount of urease coupled per 100 mg of vermiculite was 0.5163 mg (method 1) and 0.337 mg (method 2) respectively. The specific activity retained were 89% (method 1) and 80% (method 2) respectively (Table 4.6)

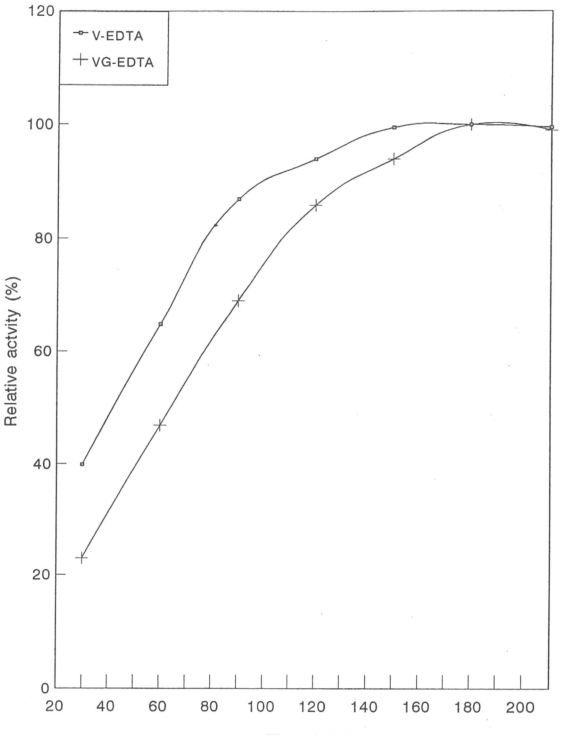


Fig. 4.14. Effect of incubation period on the activity of urease immobilized on vermiculite-EDTA (V-EDTA) and vermiculite-glutaraldehyde-EDTA (VG-EDTA). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

**Table 4.6:** Analysis of immobilized urease

| Coupling Method No. | Specific activity (mM/min/mg protein) | Protein bound to vermiculite (mg/100mg) | Retained activity (%) |
|---|---|---|---|
| 1 | 26.27 | 0.5159 | 82 |
| 2 | 28.433 | 0.347 | 89 |

*100 mg vermiculate and 1 mg/ml urease were used in each emzperiment

### 4.3.2 Properties of soluble and immobilized urease

Figure 4.15 shows the effects of temperature on free and immobilized urease. The highest relative activity of immobilized urease was obtained by both Methods 1 and 2 at 65°C. The activity of free urease was found to reach a maximum at 25°C. The significant increase in the optimum temperature when urease was bound to vermiculite indicated that immobilized urease resisted denaturation due to temperature rise. Similar observation was made by other workers (Iyengar and Rao, 1979; Lai and Tabatabai, 1992) using urease bound to chitin.

Vermiculite is a known thermal insulator. The increased adsorption of protein with increasing temperature was reported to be due to the unfolding of the protein molecules, having more functional groups available for binding to the support (Weetall and Messing, 1972). Tarafdar (1982), reported that the increase of temperature increased the adsorption of urease, acid and alkaline phosphatases

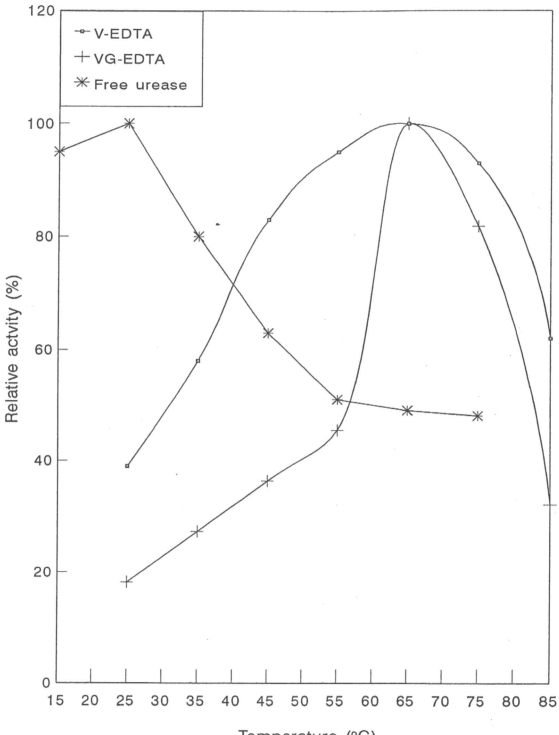


Fig. 4.15. Effect of temperature on the activity of urease immobilized on vermiculite-EDTA (V-EDTA) and vermiculite-glutaraldehyde-EDTA (VG-EDTA). The enzyme assay was carried out at various temperature 15 minutes and buffer pH of 7.0.

on clay and other minerals. The optimum temperature for immobilized urease activity increased with an increase in temperature up to a certain point after which, due to enzyme denaturation, a decline in the activity was observed, as can be seen in Figure 4.15.

Figure 4.16 shows the effect of temperature on the stability of urease immobilized on vermiculite and free urease. The relative activity of free enzyme retained was found to be 30% at 60°C. The activity retained by urease immobilized by Method 1 and 2 was found to be 50% and 70% at 60°C respectively. When compared to the free enzyme, the immobilized ureases were found to have a higher thermal stability. The improved stability of the immobilized urease may be due to the preventative of auto digestion (Goldstein, 1973).

The effect of buffer pH on the activity of urease in immobilized and free state are shown in Figure 4.17. Immobilization did not shift or alter the optimum pH of the enzyme which had the value of 6.5. The optimum pH of Jackbean urease immobilized on clay mineral kaolinite and other mineral constitutents was found to be the same as that of free urease of pH 7.0 (Tarafdar, 1987). Similar observations were made by Lai and Tabatabai, (1992); and Boyd and Mortland, (1985).In general, if an enzyme is in solution, it is not stable during storage, and its activity is gradually reduced. The stabilities during storage of urease at $25^0$ C and $4^0$ C were investigated. From Figure 4.17, it can be seen that immobilized urease

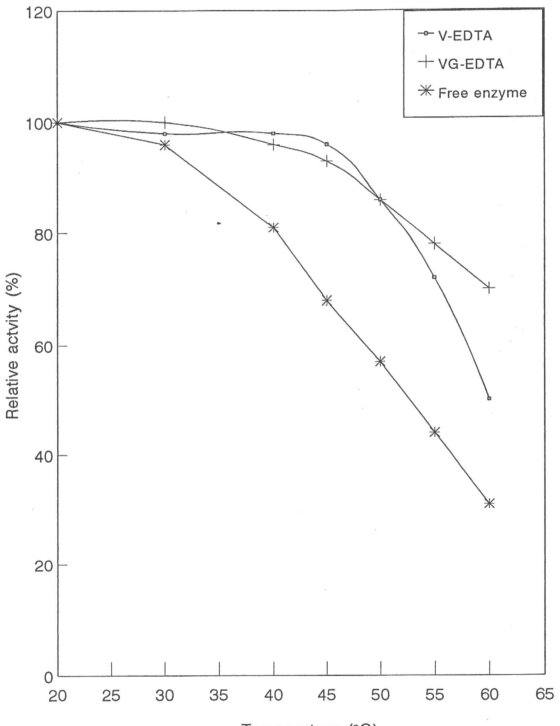


Fig. 4.16. Effect of temperature on the stability of free urease and urease immobilized on vermiculite-EDTA (V-EDTA) and vermiculite-glutaraldehyde-EDTA (VG-EDTA). The enzyme assay was carried out at various temperature for 15 minutes at buffer pH of 7.0.

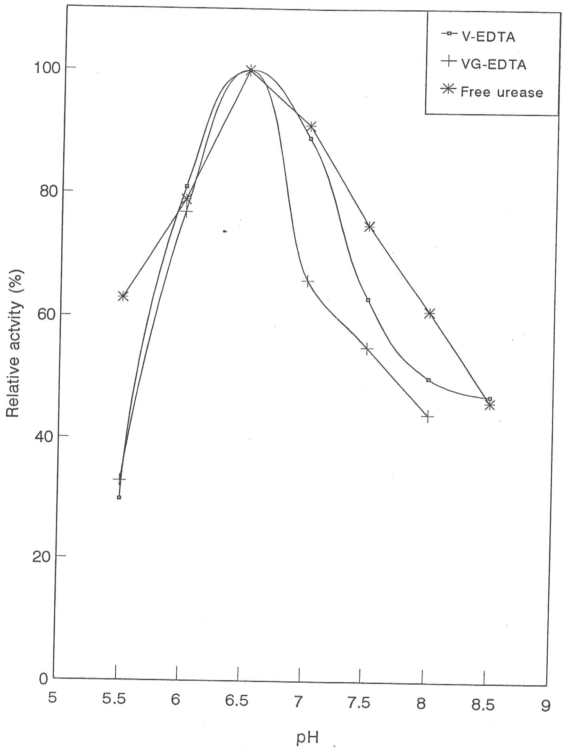


Fig. 4.17. Effect of buffer pH on the activity of free urease and urease immobilized on vermiculite-EDTA (V-EDTA) and vermiculite-glutaraldehyde-EDTA (VG-EDTA). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C. The buffer pH was varied.

obtained by Method 1 retained 69% and 30% of its original activity after 60 days when stored at 4°C and at 25°C respectively. The immobilized urease obtained by Method 2 retained 81% and 45% of its original activity after 60 days when stored at the respective temperatures of 4°C and 25°C.

Figure 4.19 shows that the maximum activity of immobilized urease and free urease obtained by both Methods 1 and 2 were obtained at substrate urea concentration of 4%.

When enzyme reactions occur in solution, even if active enzyme remains after the reactions, the desired constituent is removed and the residual enzyme is deactivated. Therefore, the reusability of the immobilized urease for urea hydrolysis is of significance. In the present work, employing Methods 1 and 2, it was possible for immobilized urease to retain 68% and 57% of its original activity after 5 repeated uses respectively (Table 4.7).

The effect of substrate concentration on urease activity expressed in Lineweaver-Burk plots are shown in Figure 4.20. The michaelis constant (Km) values of immobilized and free urease wree evaluated from the reciprocal plots of enzyme activity versus concentration (w/v) (mM) of urea. As shown in Table 4.8, the Km value of free urese was lower than of urease immobilized by Methods 1 and 2 and the Km value of urease immobilized by Method 2 was lower than that of

urease immobilized by Method 1. The method of immobilization of urease on vermiculite altered its Km value.

**4.7 :** Reusability of immobilized urease on Vermiculite

| **Number of times used** | **Retained activity(%) Method 1** | **Retained activity(%) Method 2** |
|---|---|---|
| 1 | 92 | 88 |
| 2 | 87 | 79 |
| 3 | 80 | 70 |
| 4 | 75 | 61 |
| 5 | 68 | 57 |

**Table 4.8** : Km and $V_{max}$ values of free and immobilized urease from the Lineweaver-Burk plots of the Michaelis - Menten equation.

| Urease | Km | $V_{max}$ |
|---|---|---|
| Free | 3.125 | 16 |
| Immobilized on vermiculite -glutaraldehyde-EDTA | 4.167 | 1.45 |
| Immobilized on vermiculite -EDTA | 4.76 | 2.29 |

Km expressed in mM
$V_{max}$ expressed in mm of ammonia released/min

The affinity of the enzyme towards the substrate urea was evaluated as Km values. From the Km values obtained, it is clear that free urease has a greater affinity for urea than immobilized urease. It was also found that the $V_{max}$ values of urease immobilized on vermiculites by both the methods were lower than those of free urease.

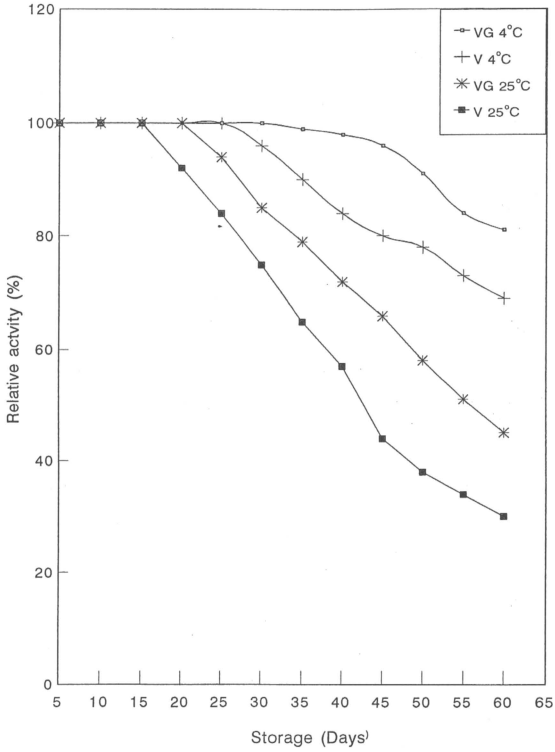


Fig. 4.18. Storage stability of urease immobilized on vermiculite-EDTA (V $4^0$ C, V $25^0$ C) and vermiculite-glutaraldehyde-EDTA (VG $4^0$ C, VG $25^0$ C) at different temperatures. The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

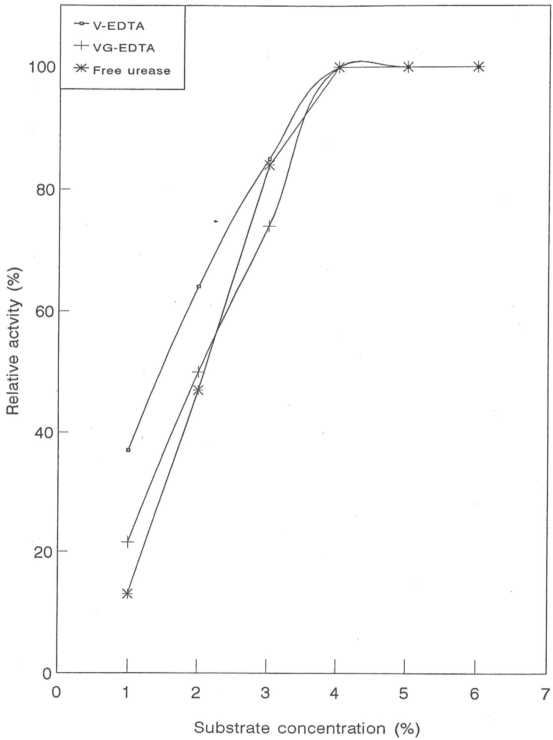


Fig. 4.19. Effect of substrate concentration on the activity of free urease and urease immobilized on vermiculite-EDTA (V-EDTA) and vermiculite-glutaraldehyde-EDTA (VG-EDTA). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

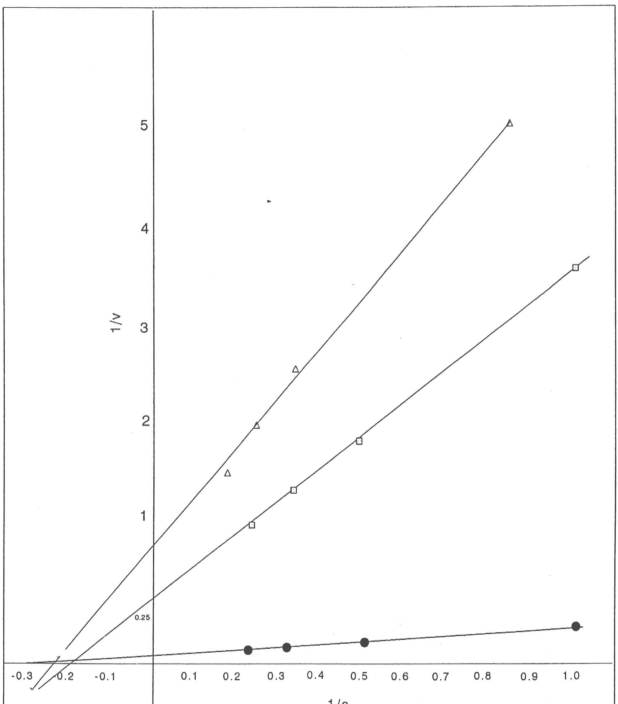


Fig 4.20. Lineweaver-Burk plots for free and immobilized urease activity. The substrate (S) is in mM, velocity (V), Urease immobilized on vermiculite-glutaraldehyde-EDTA (△) on vermiculite-EDTA (□), Free enzyme (●) are used in these studies.

## 4.4 IMMOBILIZATION OF UREASE ON AMBERLITE MB-1

### 4.4.1 Studies on immobilization parameters

As seen from Figure 4.21, the optimum pH for urease immobilized by Amberlite MB-1 and glutaraldehyde was 6.0. A small shift from the optimum pH was found to cause significant reduction in the relative activity of the immobilized enzyme. These results are in agreement with the observation made by other workers (Kobayashi *et al.*, (1992).

When the concentration of urease was increased during immobilization, the relative activity of the immobilized enzyme was found to increase (Figure 4.22). Urease concentration of 7 mg/ml was found to give the optimum level of activity. Above these urease concentrations, there was no change in the relative activity. To optimize the enzyme immobilization procedure it is important to determine the capacity of the support for the highest retention of enzyme activity.

As seen in Figure 4.23, the carrier Amberlite MB-1 used was found to reach saturation within 4 hours of incubation at 4°C. Beyond this, there was no further increase in the relative activity. This represents an improvement over the value reported by Jiugao *et al.* (1994), who found that immobilization was most effective when the reacting time was 24 hours. However, Tarafdar *et al.* (1982) found urease to be quickly adsorbed on clay surfaces and equilibrium was attained within one hour of incubation.

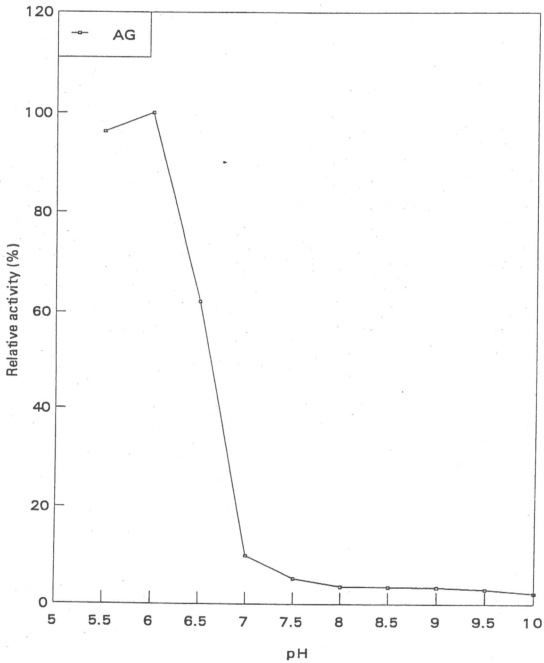


Fig 4.21. Effect of immobilization pH on the activity of urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^{0}$ C and buffer pH of 7.0.

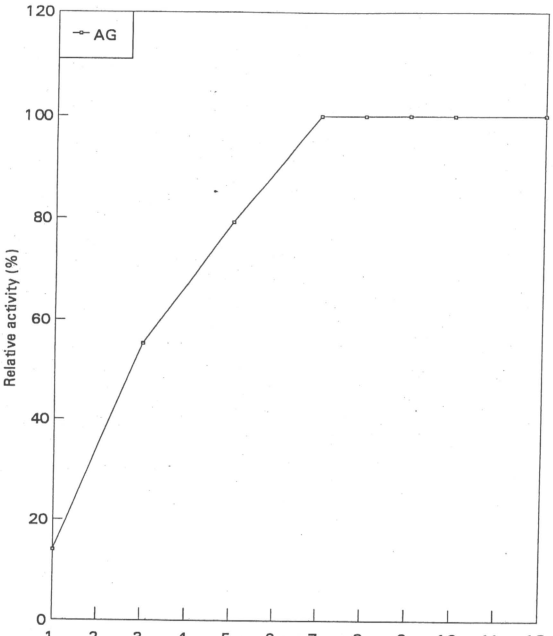


Fig 4.22. Effect of enzyme amount on the activity of urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of 55° C and buffer pH of 7.0.

### 4.4.2 Properties of soluble and immobilized urease

Figure 4.24 illustrates the effects of temperature on free and immobilized urease where the highest relative activity of immobilized urease was obtained at 75°C. The activity of free urease was found to be maximum at 25°C. The significant increase in the optimum temperature when urease was bound to Amberlite MB-1 indicated that immobilized urease resisted denaturation due to the unfolding of the protein molecules, having more functional groups available for binding to the support (Weetall and Messing, 1972). Similar observations were made by other workers (Krajewska *et al.*, 1990; lai and Tabatabai, 1992). The optimum temperature for immobilized urease activity increased with the increase in temperature up to a certain point after which, de to denaturation of the enzyme, a decline in the activity was observed.

The effect of temperature on the stability of urease immobilized on Amberlite MB-1 and free urease is shown in Figure 4.25. At 60°C, the activity of free enzyme retianed was found to be 30% and the activity retained by immobilized urease was found to be 74%. The immobilized urease was found to have higher thermal stability when compared to that of the free enzyme. Martinek *et al.* (1973), demonstrated that the thermostability of enzymes may be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint fashion.

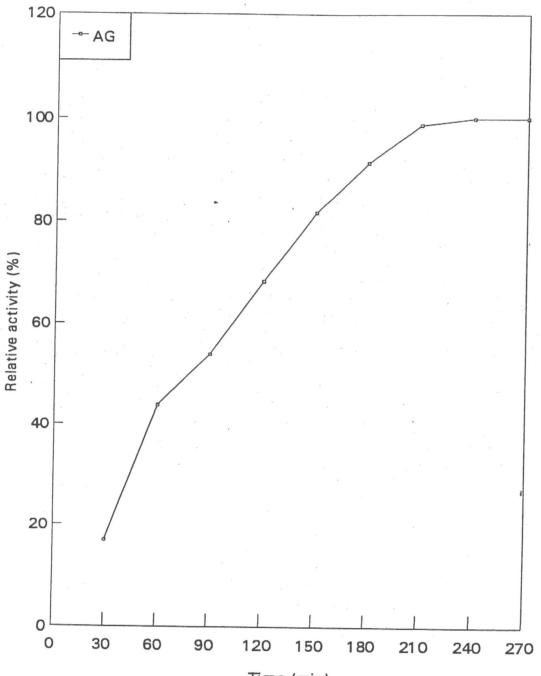


Fig 4.23. Effect of incubation period on the activity of urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of 55° C and buffer pH of 7.0.

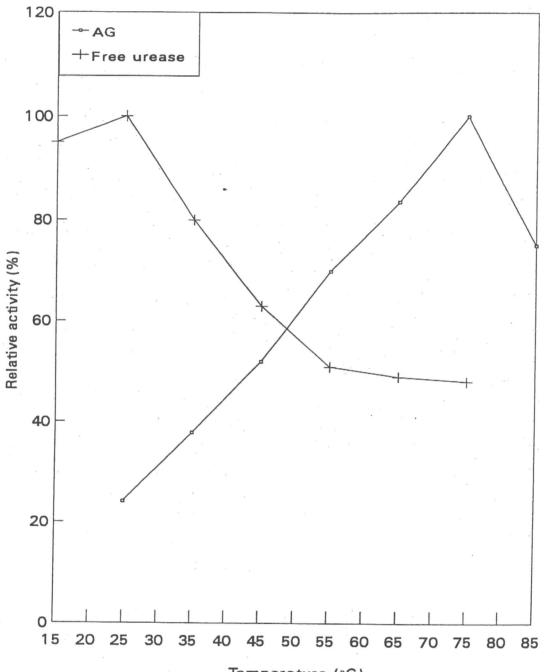


Fig 4.24. Effect of temperature on the activity of free urease and urease immobilized on Amberlite (AG). The enzyme assay was carried out various temperatures for 15 minutes at buffer pH of 7.0.

The pH effect on the activity of urease in immobilized and free state is shown in Figure 4.26. Immobilization did not shift or alter the optimum pH of the enzyme. The optimum pH for both the immobilized urease and free urease was 6.5. Similar observations were made by Boyd and Mortland (1985).

As shown in Figure 4.27, immobilized urease retained 62% and 25.3% of its original activity after 60 days when stored at 4°C and 25°C respectively. Various other reports confirmed that the storage stability of immobilized urease varies depending on the immobilization method applied (Raghunath *et al.*, 1984; Madeira, 1977; Ramachandran and Perlmutter, 1976; Sundaram and Hornby, 1970).

Figure 4.28 shows that the maximum activities of immobilized urease and free urease were obtained at substrate urea concentration of 4%.The reusability of the immobilized urease for urea hydrolysis is very important. The immobilized urease retained 65% of its original activity after 5 repeated uses respectively.

The maximum retained activity of 59% was found for urease immobilized on Amberlite MB-1. This activity represents an improvement over the value reported by other investigators 51% (Rieseland Katchalski, 1964), 20-30% (Iyengar and Rao, 1979) and 29.6%, 19.9% (Karube and Suzuki, 1972).

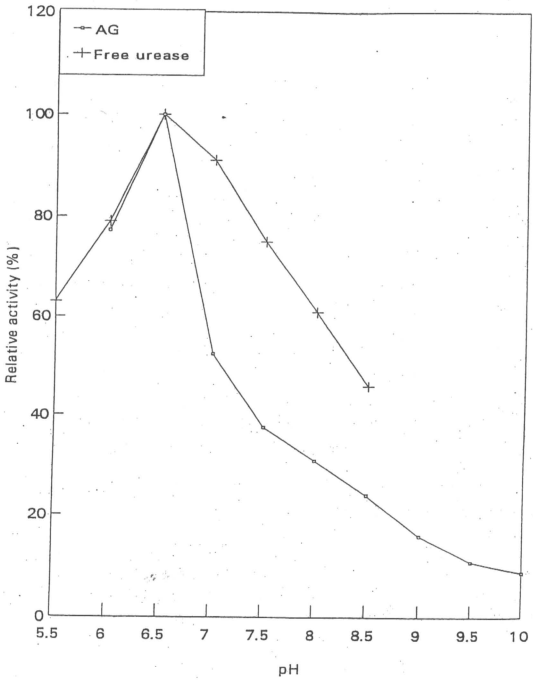


Fig 4.25 Effect of temperature on the stability of free urease and urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of 55° C and buffer pH of 7.0.

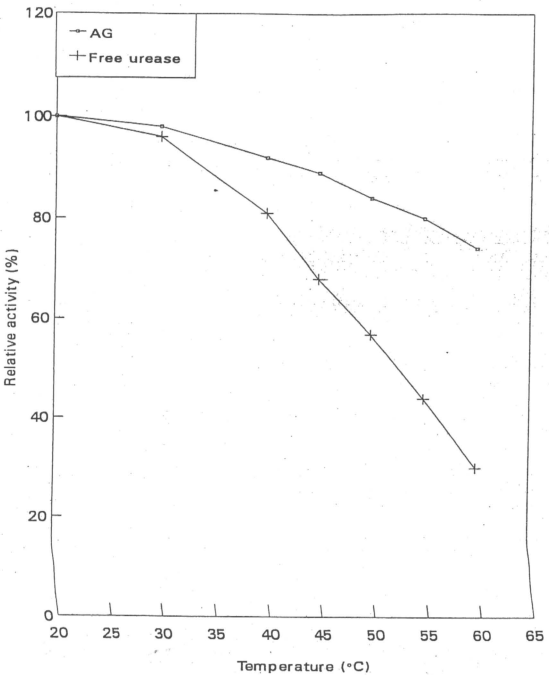


Fig 4.26 Efect of buffer pH on the activity of free urease and urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of 55° C. The buffer pH was varied.

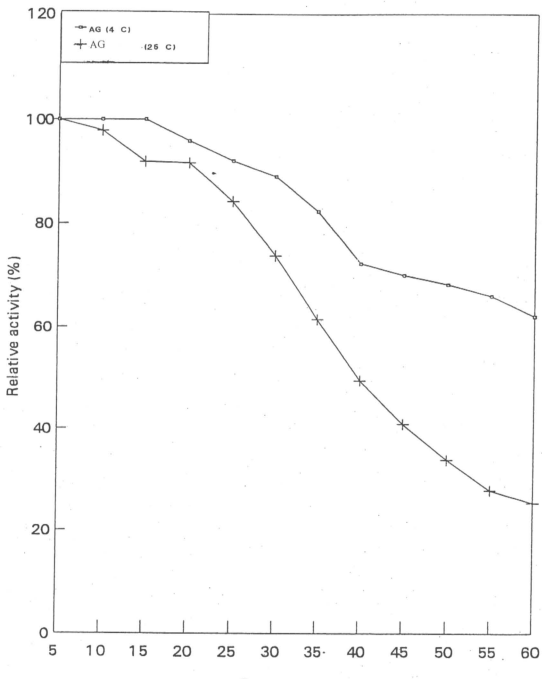




Fig. 4.27. Storage stability of urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of 55° C and buffer pH of 7.0.

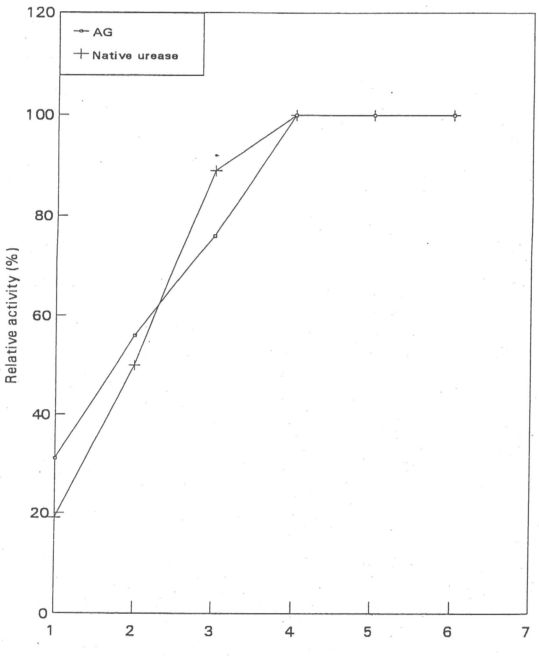


Fig.4.28. Effect of substrate concentration on the activity of free urease and urease immobilized on Amberlite (AG). The enzyme assay was carried out for 15 minutes at incubation temperature of $55^0$ C and buffer pH of 7.0.

**Table 4.9 : Reusability of immobilized urease on Amberlite MB-1**

| Number of times used | Retained activity(%) |
|---|---|
| 1 | 92 |
| 2 | 87 |
| 3 | 80 |
| 4 | 75 |
| 5 | 68 |

The kinetic results obtianed for the free and immobilized urease are presented in Figure 4.29 as Lineweaver-Burk plots 1/V versus 1/[s]. The linear nature of the Lineweaver-Burk plots proves that, in the range of urea concentration examined, both the enzymes follow the Michaelis-Menten kinetics represented by the following equation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{Km}{V_{max}} \frac{1}{[s]}$$

where V and $V_{max}$ are the actual and maximum reaction rates respectively, Km is the Michaelis constant and [s] is the substrate concentration. The Km values of immobilized and free urease were evaluated from straight line of the reciprocal plots of the enzyme activity versus concentration (w/v) (mM) of urea as a substrate. The Km value of free urease of 3.125mM was found to be lower than that of the immobilized urease of 25.32mM. The Amberlite MB-1 immobilized urease exhibited Km value which is about 8 times higher than that of the free

urease. This increase may be a consequence of either structural changes in the enzyme introduced by the applied immobilization procedure or the lower than those from free urease (Boyd and Mortland, 1985). Increased Km of urese after immobilization has been reported by various authors (Epton *et al.*, 1974; Onyezili and Onitiri, 1981).

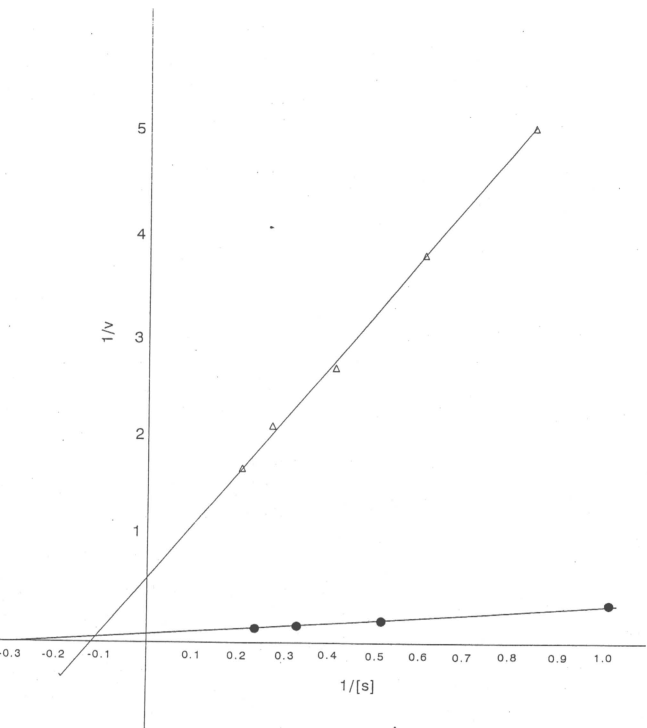


Fig 4.29: Lineweaver-Burk plots for free and immobilized urease activity. The substrate (S) is in mM, velocity (V), Urease immobilized on Amberlite (△),Free enzyme (●) are used in these studies.