CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Commercial grade Nylon 6/6 tubes were obtained from BHD Chemicals, England. Commercial grade vermiculite was obtained from M/s Tamilnadu Minerals Corporation Ltd., India while Amberlite MB-1, urea, Nessler's reagent, Jack beans urease (EC. 3.5.1.5) type IV (activity 40,000-80,000 units per gram) and glutaraldehyde were obtained from Sigma Chemical Co., U.S.A. Other reagents used in these experiments are all of analytical grade.

3.2 ESTIMATION OF UREASE ACTIVITY

The activity of urease was estimated by the method suggested by Jayaraman (1981). As the conversion of urea to ammonia by urease is stoichiometric, urease activity is estimated by measurement of ammonia formed using Nessler's reagent.

3.2.1 UREASE ACTIVITY OF A SOLUBLE ENZYME

1.0 ml of the substrate solution (2% urea solution) of pH 7.0 was added to a test tube followed by 1.0 ml of 0.1M phosphate buffer (pH 7.0). Then 1.0 ml of appropriately diluted enzyme solution was added and incubated at 55°C for 15 minutes, at the end of which it was quickly placed in ice. 1.0 ml of 0.6 N sulphuric acid was added to stop the reaction. Appropriate dilution were made to the supernatant in order to accomodate the absorbance reading within the range of the standard curve. To this 1.0 ml of the Nessler's reagent was added and after mixing, the color intensity was measured at 500 nm. The ammonia content was then read off from the standard curve. Results were multiplied by the appropriate dilution factor to obtain the ammonia concentration in the original sample. The enzyme activity is expressed in mMoles ammonia per minute per mg protein, while the relative activity was then as the amount of ammonia (mMoles) liberated expressed in percentage. To make sure that the reaction was not urea-limited, the concentration of urea used was higher than the estimated Michaelis constants.

3.2.2 UREASE ACTIVITY OF IMMOBILIZED UREASE

The activity of immobilized enzyme was determined by incubating 100 mg of immobilized enzyme in 1 ml of phosphate buffer (pH 7.0) with 1 ml of 2% urea solution (pH7.0) at 55°C for 15 minutes. From the above mixture, the filtrate was

taken and colorimetrically measured for activity at 500 nm after developing color with Nessler's reagent as described in section 3.2.1.

3.3 PROTEIN ESTIMATION

Protein determination of soluble urease was performed using Sigma protein assay kit. The procedure performed was based on Peterson's modification of the Micro-Lowry method (1951).

Protein estimation of the soluble urease is necessary, to estimate the amount of protein (urease) bound to the support. The quantity of protein bound on the support was calculated by substracting the protein recovered in the combined washings of the support urease complex from the protein used for immobilization. The protein concentration of the urease used for immobilization and those recovered in the washings are determined from the standard curve drawn with the protein standard solutions using the Sigma protein assay kit comprising of Lowry Reagent, protein standards and Folin and ciocalteau's phenol reagent. An alkaline cupric tartrate reagent complexes with the peptide bonds of the protein (urease) and forms a purple-color when the phenol reagent is added. The amount of protein in the samples was measured colorimetrically, using a spectrophometer.

3.3.1 PROTEIN ESTIMATION OF THE SAMPLE

Sample urease solutions and washings was added to labelled test tubes and diluted to 1.0 ml with water. Appropriate dilutions were made to the sample in order to accommodate the absorbance reading within the range of the standard curve. 1.0 ml of Lowry Reagent solution was added to the sample tubes and mixed well. The solutions were allowed to stand at room temperature for 20 minutes. Then with rapid mixing, 0.5 ml Folin ciocalteu's Phenol Reagent Working Solution was added and allowed colour to develop for 30 minutes. Finally, the solutions were transferred to cuvettes and absorbance of the sample tubes at wavelength 750 nm were measured. The protein content of the samples was then read off from the standard curve constructed with Protein Standard Solution. Results were multiplied by the appropriate dilution factor to obtained the protein concentration in the original sample.

3.4 IMMOBILIZATION OF UREASE

3.4.1 IMMOBILIZATION OF UREASE ON NYLON

3.4.1.1 Preparation and activation of Nylon

Commercial grade nylon 6/6 tubes of one gram were treated as reported by Chellapandian and Sastry (1994) with 20 ml of 3.5M hydrochloric acid at 50°C for 30 minutes. The partially hydrolyzed nylon was washed with distilled water. Before coupling the enzyme, the partially hydrolyzed nylon was treated with glutaraldehyde.

3.4.1.2 Immobilization of urease on nylon

100 mg of Nylon was treated with 2.5% (v/v) glutaraldehyde for 15 minutes. The activated nylon was washed throughly with distilled water remove excess glutaraldehyde. The 1.5 ml of phosphate buffer (pH 7.0) and 1 mg/ml of urease solution were treated with glutaraldehyde activated nylon for 3 hours with occasional shaking. The uncoupled enzyme was removed by washing with 1M sodium chloride, distilled water and finally with phosphate buffer (pH7.0). The immobilized enzyme was stored in phosphate buffer (pH7.0) at 4°C. The immobilization parameters such as immobilization pH, enzyme concentration and enzyme coupling time were studied.

3.4.1.2.1 Effect of immobilization pH

100 mg of nylon was treated with 2.5% (v/v) glutaraldehyde for 30 minutes. The activated nylon was washed thoroughly with distilled water and then 1.5 ml of phosphate buffer (pH 5.5 to 10.0) and 1 mg/ml of urease solution were added to the glutaraldehyde activated nylon and was then kept for 3 hours with occasional shaking at room temperature. The buffer pH was varied from 5.5 to 10.0 by the addition of 0.1M sodium hydroxide or hydrochloric acid. The uncoupled enzyme was recovered by washing with 1M sodium chloride. The immobilized enzyme was assayed as described earlier in Section 3.2.2.

3.4.1.2.2 Effect of enzyme amount

The nylon-glutaraldehyde conjugate were treated with 1.5ml of phosphate buffer of pH 6.0 and 1ml of urease solution for 3 hours with occasional shaking at room temperature. The quantity of enzyme was varied from 1mg/ml to 12mg/ml. The excess and uncoupled enzyme was removed and assayed as described earlier in Section 3.2.2.

3.4.1.2.3 Effect of time on urease immobilization

The nylon-glutaraldehyde conjugate were treated with 1.5 ml of phosphate buffer of pH6.0 containing 7 mg/ml of urease and allowed to react for different periods of time. The enzyme coupling time was varied up to 270 minutes. Then the excess and uncoupled enzyme was removed and immobilized enzyme was assayed as described earlier in Section 3.2.2.

3.4.1.3 Properties of soluble and immobilized urease

The properties of immobilized urease and free urease such as effect of temperature, thermal stability, effect of buffer pH, optimum substrate concentration, storage stability and reusability were also studied

3.4.1.3.1 Effect of temperature

1.0ml of the substrate solution (2% urea) and 1.0ml of 0.1M phosphate buffer (pH 7.0) were added to 100mg of immobilized enzyme and 1mg/ml of free enzyme solution respectively and incubated at various temperature (15^0 C to 85^0 C) for 15 minutes. Then the filtrate was taken and measured for activity at 500nm after developing color with Nessler's reagent as described in Section 3.2.1.

3.4.1.3.2 Determination of thermal stability

Thermal stability of the free and immobilized urease was evaluated by measuring the residual activity of urease exposed to various temperatures in 0.1M phosphate buffer of pH 7.0 for 15 minutes. After heating, the samples were quickly cooled to 55^oC and assayed for enzyme activity immediately as previously described in Section 3.2.1 and 3.2.2. The remaining activities were expressed as relative to the original activities assayed at 55^oC without heating.

3.4.1.3.3 Effect of buffer pH

The assays for free and immobilized urease activities were carried out as previously described in Section 3.2.1 and 3.2.2 respectively except the buffer pH which was varied from 6.0 to 10.0 by the addition of 0.1M sodium hydroxide or hydrochloric acid. The incubation temperature was maintained at 55^0 C for both free and immobilized enzyme. The concentration of the substrate was 2% urea solution.

3.4.1.3.4 Storage stability

The immobilized urease was stored in phosphate buffer of pH 6.0 at 4°C and 25°C for 60 days. At frequent intervals the activity was measured as described in Section 3.2.2. The pH of the reaction mixture was maintained at pH 7.0 and incubation temperature at 55^0 C.

3.4.1.3.5 Determination of optimum substrate concentration

The assays of free and immobilized urease activities were carried out as previously described in Section 3.2.1 and 3.2.2 respectively except by varying the substrate concentration from 1% to 6%. The pH of the reaction mixture was maintained at pH 7.0 and incubation temperature at 55^0 C for both free and immobilized enzyme.

3.4.1.3.6 Reusability

The immobilized urease was repeatedly used for the hydrolysis of urea at standard conditions.

3.4.2 ADSORPTION OF UREASE ON VERMICULITE SATURATED WITH DIFFERENT CATIONS

3.4.2.1 Preparation of cationic vermiculite

Commercial grade vermiculite was treated as reported by Chellapandian and Sastry (1993) with 1N sodium acetate-acetic acid buffer of pH 5. The excess buffer was removed by centrifugation. Residual vermiculite was further treated with 30% hydrogen peroxide to remove organic matter present. Iron oxide present in the material was removed by dithionite-citrate method (Jackson, 1967). The free amorphous silica and alumina were removed by boiling the sample in 2% sodium carbonate solution. Vermiculite so prepared was washed with distilled water.

Cationic vermiculite was prepared by adding 1M solution of chlorides of calcium, potassium and aluminium to the treated vermiculite. The excess chloride was removed by repeated washing with 80% methanol.

3.4.2.2 Adsorption of urease on vermiculite

Adsorption of urease was carried out by interacting suitable quantity of vermiculite with a known amount of enzyme. Aluminium, calcium and potassium saturated vermiculite suspension of 100 mg as prepared above in Section 3.4.2.1 was treated with 1 mg/ml enzyme solution (pH 7.0) of known activity. The samples were equilibrated at 45^0 C for 3 hours and centrifuged to separate the

adsorbent from suspension. Finally, the adsorbent was washed 3 times with distilled water to remove the loosely bound or unbound enzyme. The final washings were analyzed for protein according to the method developed by Lowry et al. (1951) to confirm the completeness of washing off loosely bound enzyme. The amount of enzyme present in the supernatant was assayed as described in Section 3.2.1. The difference between initial enzyme units added and enzyme units present in the supernatant indicates the amount of enzyme adsorbed on the vermiculite. The parameters involved in the adsorption such as temperature effect, adsorbent dose, enzyme amount and equilibrium time were studied.

3.4.2.2.1 Equilibrium time

The three types of cationic vermiculites were brought into contact with urease (1 mg/ml) incubated at 45°C. The amount of enzyme bound to the cationic vermiculite was determined periodically up to 4 hours. A minimum time required to reach equilibrium was determined by this experiment.

3.4.2.2.2 Effect of temperature

To determined the effect of temperature on adsorption of enzyme, urease (1 mg/ml) was brought into contact with cationic vermiculites at different temperatures for 3 hours. The temperature was varied between 20°C to 70°C.

3.4.2.2.3 Effect of pH

To determine the adsorption efficiency in relation to pH of the medium, 0.1M sodium phosphate buffers of different pH were added to 100 mg of cationic vermiculite and incubated for 3 hours at 45^0 C.

3.4.2.2.4 Effect of dose of cationic vermiculite

To detemine the optimal cationic vermiculite dose, separate experiments were conducted with varying doses of vermiculite in contact with the same amount of urease for 3 hours at 45°C. The doses of vermiculite were varied from 10, 20, 40, 60,70,80 to 120 mg/ml.

3.4.2.2.5 Effect of enzyme concentration

This study was conducted by varing the quantities of enzyme added, keeping the temperature at 45^0 C, cationic vermiculite dose at 100mg and incubation time of 3 hours.

3.4.2.2.6 Storage stability

Immobilized urease was stored in 0.1M sodium phosphate buffer (pH 6.0) at 4°C for 60 days. At regular intervals the activity was measured.

3.4.3 IMMOBILIZATION OF UREASE ON VERMICULITE

3.4.3.1 Preparation of vermiculite

Commercial grade vermiculite was treated as reported earlier in Section 3.4.2.1 except that only one type of cationic vermiculite was prepared by adding 1M aluminium chloride solution to the treated vermiculite. The excess chloride was removed by repeated washing with 80% methanol till the supernatant liquid was free from chloride.

3.4.3.2 Immobilization of urease

3.4.3.2.1 Method 1: Vermiculite + EDTA + Urease

100 mg of vermiculite was immersed in ice-cold buffer solution containing 1 mg/ml enzyme and the mixture was kept at 4 C overnight. Urease was coupled with vermiculite in 0.1M sodium phosphate with 0.0078g EDTA at pH 6.0. The vermiculite bound urease was separated from the unbound urease by centrifugation and washed several times with 1M sodium chloride, distilled water and finally with a buffer solution of 0.1M sodium phosphate (pH 6.5).

3.4.3.2.2 Method 2 : Vermiculite+ Glutaraldehyde + EDTA + Urease

100 mg of vermiculite was treated with 2.5% (v/v) glutaraldehyde for 15 minutes. Vermiculite-glutaraldehyde conjugate was washed with distilled water to

remove excess glutaraldehyde. The conjugate was immersed in ice-cold buffer solution containing 1 mg/ml enzyme and the mixture was kept at 4 C overnight. Urease was coupled in 0.1M sodium phosphate with 0.0078g EDTA at pH 6.0. The vermiculite-glutaraldehyde-EDTA-urease conjugate was centrifuged and washed several times with 1M sodium chloride, distilled water and finally with a buffer solution of 0.1M sodium phosphate (pH 6.5).

3.4.3.2.3 Effect of immobilization pH

The vermiculite (method 1) and vermiculite-glutaraldehyde conjugate (method 2) was treated as described earlier in Section 3.4.3.2.1 and 3.4.3.2.2 respectively except the buffer pH was varied from pH 5.5 to pH 9.0 by the addition of 0.1M sodium hydroxide or hydrochloric acid and kept at 4^0 C overnight. The vermiculite-bound urease was separated from the unbound urease by centrifugation and was washed several times with 1M sodium chloride, distilled water and finally with 0.1M sodium phosphate (pH 6.5) and was assayed as described earlier in Section 3.2.2.

3.4.3.2.4 Effect of urease concentration on immobilization

The vermiculite (method 1) and vermiculite-glutaraldehyde conjugate (method 2) was mixed with 1ml of phosphate buffer containing urease of various concentration and kept at 4^0 C overnight. The pH of the phosphate buffer was maintained at pH 6.0 and pH 5.5 for vermiculite and vermiculite-glutaraldehyde

conjugate respectively. The immobilized enzyme was assayed as described earlier in Section 3.2.2.

3.4.3.2.5 Effect of time on urease immobilization

The vermiculite (method 1) and vermiculite-glutaraldehyde conjugates (method 2) were treated with 1 ml of phosphate buffer of pH 6.0 and pH 5.5 respectively containing 1 mg/ml of urease, and stored at 4^0 C for different periods of time. The immobilized enzyme was assayed as described earlier in Section 3.2.2.

3.4.3.3 Properties of soluble and immobilized urease

The properties of immobilized urease and free urease such as effect of temperature, thermal stability, storage stability, optimum substrate concentration, effect of buffer pH and reusability were also studied.

3.4.3.3.1 Effect of temperature

The activity of free and immobilized urease was assayed as previously described in Section 3.2.1 and 3.2.2 respectively, at increasing temperatures (15 C to 85 C) for 15 minutes. The substrate and buffer pH was maintained at pH 7.0.

3.4.3.3.2 Determination of thermal stability

Thermal stability of the free and immobilized urease was evaluated as previously described in Section 3.4.1.3.2.

3.4.3.3.3 Effect of buffer pH

The assay of free and immobilized urease activities were carried out under standard conditions as described in Section 3.2.1 and 3.2.2 respectively. The substrate and buffer pH was varied from pH 5.5 to pH 8.5 by the addition of 0.1M sodium hydroxide or hydrochloric acid. The incubation temperature was maintained at 55^0 C for both soluble and immobilized enzyme.

3.4.3.3.4 Storage stability

The immobilized urease of method 1 and method 2 were stored in phosphate buffer of pH 6.0 and 5.5 respectively at 4°C and 25°C for 60 days. Its activity was measured at frequent intervals as described earlier in Section 3.2.2.

3.4.3.3.5 Determination of optimum substrate concentration

The assays of free and immobilized urease activities were carried out under standard conditions as described in Section 3.2.1 and 3.2.2 except the substrate concentration was varied from 1% to 6% for both free and immobilized enzyme. The pH of the reaction mixture was maintained at pH 7.0 and incubation temperature at 55^0 C for both free and immobilized enzyme.

3.4.3.3.6 Reusability

The immobilized urease was repeatedly used for the hydrolysis of urea at standard conditions.

3.4.4 IMMOBILIZATION OF UREASE ON AMERLITE MB-1

3.4.4.1 Immobilization of urease

The procedure described by Chellapandian and Sastry (1993) ws adopted. 100 mg of Amberlite MB-1 was treated with 2.5% (v/v) glutaraldehyde for 15 minutes. In this study, the Amberlite-glutaraldehyde then 1.0 ml of phosphate buffer (pH 6.0) and 1 mg/ml urease solution was treated with glutaraldehyde activated Amberlite MB-1 for 4 hours. The uncoupled enzyme was removed by washing with 1M sodium chloride, distilled water and finally with phosphate buffer (pH 6.5) and the immobilized enzyme was then stored in phosphate buffer (pH 6.0) at 4°C.

3.4.4.1.1 Effect of immobilization pH

The Amberlite-glutaraldehyde conjugate was treated as described above in Section 3.4.4.1 except the buffer pH was varied from pH 5.5 to pH 10.0 by the addition of 0.1M sodium hydroxide or hydrochloric acid and kept for 4 hours. The quantity of enzyme was maintained at 1 mg/ml and incubation period of 4 hours. The immobilized enzyme was assayed as described earlier in Section 3.2.2.

3.4.4.1.2 Effect of urease concentration on immobilization

The Amberlite-glutaradehyde conjugate was mixed with 1ml of phosphate buffer of pH 6.0 containing urease of various concentration and allowed to react for 4 hours. The immobilized enzyme was assayed as described in Section 3.2.2.

3.4.4.1.3 Effect of time on urease immobilization

The Amberlite-glutaraldehyde conjugates were treated with 1 ml of phosphate buffer of pH 6.0 containing 7 mg/ml of urease and allowed to react for different periods of time. The immobilized enzyme was assayed as described in Section 3.2.2.

3.4.4.2 Properties of soluble and immobilized urease

The properties of immobilized urease such as effect of temperature, thermal stability, storage stability, optimum substrate concentration, effect of buffer pH and reusability were also studied.

3.4.4.2.1 Effect of temperature

The activity of free and immobilized urease was assayed as previously described in Section 3.2.1 and 3.2.2 respectively, at increasing temperatures (15^0 C to 85^0 C) for 15 minutes. The substrate and buffer pH was maintained at pH 7.0.

3.4.4.2.2 Determination of thermal stability

Thermal stability of the free and immobilized urease was evaluated as previously described in Section 3.4.1.3.2.

3.4.4.2.3 Effect of buffer pH

The assays for free and immobilized urease activities were carried out as previously described in Section 3.2.1 and 3.2.2 respectively. The substrate and buffer pH was varied from pH 5.5 to pH 10.0 by the addition of 0.1M sodium hydroxide or hydrochloric acid. The incubation temperature was maintained at 55^0 C for both soluble and immobilized enzyme.

3.4.4.2.4 Storage stability

The immobilized urease was stored in a phosphate buffer of pH 6 at 4°C and 25°C for 60 days. Its activity was measured at frequent intervals at standard conditions as described in Section 3.2.2.

3.4.4.2.5 Determination of optimum substrate concentration

The assays for free and immobilized urease activities were carried out under standard conditions as described in Section 3.2.1 and 3.2.2 except the substrate concentration was varied from 1% to 6%.

3.4.4.2.6 Reusability

The immobilized urease was repeatedly used for the hydrolysis of urea at standard conditions.