Activity and stability of immobilized urease on different matrices (nylon-6, 6 beads, sepharose gel, silica gel and gelatin film coated on cellulose acetate membrane) have been investigated. Different covalent coupling methods using ascorbic acid or glutaraldehyde for polyamine matrices and periodate for polyhydroxy matrix and tosyl chloride for silica gel were used. A relatively less expensive source of urease (crude extract of jack bean meal) was used. Periodate oxidized sepharose CL 6B gel was able to retain 74% of the enzyme on immobilization while ascorbic acid coupling to gelatin film retained 66%. Immobilized urease on nylon beads and gelatin film stored in 50 mM phosphate buffer (pH 8) at 4°C showed practically no leaching of the enzyme retaining activity over a period of 30 days, while urease coupled to sepharose and silica gel stored under the same conditions was stable over a period of 10 days. Immobilized urease retained activity over a wide pH range and was more stable than the free enzyme at lower and higher pH values. Immobilized urease was also more stable than the free enzyme over a wide temperature range. Nylon bead immobilized jack bean urease had a higher $K_m$ (0.62 mM) than that of the soluble enzyme (0.55 mM). Kinetic studies on the hydrolysis of urea using immobilized urease in batch and fixed bed flow reactor configurations have been carried out.

Keywords: Ascorbic acid coupling, Enzyme immobilization, Urease activity, Urease immobilized on gelatin film

Introduction
Urea has a considerable significance in clinical chemistry in kidney disease and renal failure, besides agricultural chemistry for determination of urea in fertilizers, for determination of water quality, and in seawater analysis. Urease (urea aminohydrolase, EC 3.5.1.5), a nickel dependent metalloenzyme, catalyses the hydrolysis of urea to form ammonia and carbon dioxide. Removal of excess urea has been a major problem for patients suffering from renal failure. Urease has been used in an immobilized form in kidney machines for blood detoxification. Immobilization of urease has been carried out on several matrices for clinical analytical applications, and has also been used for the treatment of urea-containing effluents. An acid urease from *Arthrobacter mobilis* has been used for removal of urea from fermented beverages, such as sake. Polysaccharides have been used for the immobilization of enzymes in several studies.

Present study is a comparative evaluation of covalent coupling of urease on various matrices (nylon-6, 6 beads, sepharose gel, silica gel and gelatin film coated on cellulose acetate membrane) by different coupling methods.

Materials and Methods

**Materials**
Urease (jack bean meal) was obtained from Himedia, nylon beads used for immobilization were gifted by M/s Sriram fibers and gelatin coated on photographic film was obtained locally. Other chemicals were of analytical grade from Merck, SRL, Himedia, CDH, CHEMSPURE and SIGMA. All solutions were prepared using double-distilled water.

**Urease Assay**
Urease activity was assayed by Berthelot method with an aliquot of jack bean urease (0.1%) extract of phosphate buffer (50 mM, pH 8) containing 27 mM EDTA and sodium azide (0.1%). Urea solution (100 mM) was used as substrate. Reaction mixture was incubated for 10 min.
at 37°C. The reaction was terminated by addition of phenol-hypochlorite solution and incubated for 25 min at 55°C for colour development. Absorbance was read at 630 nm with blank solution as reference using a Systronics UV-Visible spectrometer type 118, after addition of distilled water (10 ml) for dilution. An enzyme unit is the amount of enzyme required to liberate 1 µmol of ammonia/min.

**Immobilization Methods**

**Ascorbic Acid Coupling of Urease on Nylon Beads**

Nylon beads were partially hydrolyzed with HCL (2.5 M) at room temperature for about 20 min. The beads were washed with water and immersed in ascorbic acid solution (5%) in a coupling buffer of phosphate buffer (50 mM, pH 8) containing 1 mM EDTA for 40 min. Beads collected on a filter and washed successively with water and coupling buffer, were then immersed in the crude jack bean meal extract of urease solution (0.1% in 50 mM phosphate buffer, pH 8.0) and kept overnight at 4°C. Urease immobilized nylon beads were successively washed with water, NaCl (1 M) and finally with coupling buffer. All washings were collected for protein estimation and enzyme assay. Beads were stored in 50 mM, pH 8 phosphate buffer at 4°C.

**Glutaraldehyde mediated Immobilization of Urease on Nylon Beads**

HCL activated nylon beads were immersed in coupling buffer containing glutaraldehyde (1.25% v/v in 50 mM phosphate buffer, pH 8.0) for 40 min and washed successively with water and coupling buffer. Glutaraldehyde coupled nylon beads were immersed in urease solution (0.1% in 50 mM phosphate buffer, pH 8.0) and kept overnight at 4°C followed by usual washing and storing.

**Immobilization of Urease on Sepharose Gel**

Sepharose CL 6B gel (1g) was stirred in 30 ml of phosphate buffer (50 mM, pH 7.5) containing sodium periodate (4 mg) for 60 min at 30°C. The oxidized sepharose gel was immersed in urease solution and kept overnight at 4°C followed by usual washing and storing. Gel was treated with NaBH₄ and stored in phosphate buffer (50 mM, pH 7.5).

**Coupling of Urease to Tosylchloride Activated Silica**

Column chromatography grade silica gel was washed with HCl (0.2 M) and NaOH (0.2 M) thrice alternatively and finally with distilled water until all alkali was removed. Silica was dried at 120°C for 2 h and then cooled. Gel was then washed well with dioxane and then suspended in tosyl chloride solution in dioxane (50 mg/ml), to which 0.2 ml of pyridine was added dropwise and stirred at room temperature for 2 h. Activated gel was then washed with dioxane followed by NaHCO₃ buffer (pH 8.5) and then suspended in urease solution (0.1% in 50 mM phosphate buffer, pH 8.0). The washing procedure was same as mentioned above.

**Glutaraldehyde mediated Immobilization of Urease on Gelatin Film**

Gelatin film was immersed in coupling buffer containing glutaraldehyde (1.25% v/v in 50 mM phosphate buffer, pH 8.0) for 40 min and washed successively with water and coupling buffer. Glutaraldehyde coupled gelatin film was immersed in urease solution (0.1% in 50 mM phosphate buffer, pH 8.0) and kept overnight at 4°C followed by usual washing and storing.

**Ascorbic Acid Coupling of Urease on Gelatin Film**

Gelatin film was immersed in ascorbic acid solution (5%) in a coupling buffer of phosphate buffer (50 mM, pH 8) containing 1 mM EDTA for 40 min. The film was collected on a filter and washed successively with water and coupling buffer. The film was then immersed in the crude extract of urease extract and kept overnight at 4°C. The urease immobilized gelatin film were successively washed with water, NaCl (1 M) and finally with coupling buffer. All washings were collected for protein estimation and enzyme assay. The film was stored in 50 mM, pH 8 phosphate buffer at 4°C.

**Studies on Immobilized Enzyme Activity**

Optimum pH and temperature for the immobilized urease on various matrices as well as the storage stability of immobilized enzyme were determined in batch reactor conditions. Immobilized enzyme preparation (1g) was packed into column (2.0 x 1.0 cm). A stock solution of 0.1M urea from a reservoir was allowed to pass through it. The product stream from the reactor was collected at 5 min intervals by varying flow rates.

**Results and Discussion**

**Ascorbic Acid mediated Immobilization of Urease on Poly Amino Matrices**

In the first step of surface activation of amino polymer, free amino group obtained by partial hydrolysis of polyamide (nylon 6, 6 beads) and free amino group of...
gelatin coated on cellulose acetate membrane react with ascorbic acid forming dehydroascorbic acid derivatives by oxygen dependent autoxidation. Surface activated amino polymer was washed with water and buffer. In immobilization step, activated amino polymer reacts with enzyme (Fig. 1). In this step, β- keto groups of dehydroascorbic acid derivatives of amino polymer surface react with lysine residues in enzyme forming Schiff’s base structures. Structures are not stable and form oxalic acid diamide bridge between amino polymer and enzyme due to an autoxidative fragmentation reaction.

**Immobilization Efficiency and Storage Stability**

Immobilization varied with the method / matrix as follows (Fig. 2): Periodate oxidized sepharose CL 6B gel, 74%; ascorbic acid coupling to gelatin film, 66%; ASA coupling of nylon beads, 44%; and glutaraldehyde coupling to nylon beads, 39%. Immobilized urease on nylon beads and gelatin film stored in 50 mM phosphate buffer (pH 8) at 4°C showed practically no leaching of the enzyme retaining 60% activity over a period of 30 days, while urease coupled to sepharose and silica gel stored under the same conditions was stable over a period of 10 days.

**Effect of pH and Temperature on Immobilized Urease**

An optimum pH for free enzyme activity (7-8) and immobilized urease (7-9) was found for most of the matrices. In the case of urease immobilized on sepharose and silica gel, maximum activity was found at pH 7-10, while ascorbic acid coupling of urease on nylon beads exhibited maximum activity at pH 5-9 (Fig. 3a). Activity of free enzyme was found to decrease (50%) at pH 5 and 10 compared to its maximum activity at optimum pH (7-8). In contrast, immobilized enzyme showed more activity at pH 5-10, activity decreasing only by 20% at low or higher pH values compared to the maximum activity at optimum pH. Microenvironment of enzyme
molecule is possibly modified depending on the surface and residual charges on the solid matrix and the nature of the bound enzyme due to immobilization resulting in retaining activity over an extended range of pH and also causing a shift in the pH optimum of the enzyme.

Immobilized enzyme (15-55 °C) was found to be relatively more stable than the free urease (15-40 °C) over a wider range of temperature (Fig. 3b). Free enzyme activity was found to decrease (70%) at 5°C and at 60°C from the maximum activity at 30°C. The loss in activity of immobilized enzyme was less (only 20%) under similar conditions. Similar enhanced thermal stability has been reported for several covalently bound ureases.

**Laboratory Scale Continuous Flow Reactor**

Urease immobilized on nylon beads was packed in a flow reactor. Effect of substrate flow rate on reaction rate was investigated. A flow rate dependent kinetics of urea hydrolysis by immobilized urease has been observed (Fig. 4). Increased rate of product formation at higher flow rate may be attributed to lower mass transfer inhibition as well as lower product inhibition at higher flow rate.

**Effect of Immobilization on $K_{m}$**

Urease immobilized on nylon beads showed an apparent $K_{m}$ value (0.62 mM), which is higher than the $K_{m}$ of soluble urease (0.55 mM). This may be explained on the basis of a concentration gradient of substrate established across the ‘Nernst layer’, an unstirred layer of solvent surrounding the suspended matrix particles. Consequently, saturation of an immobilized enzyme molecule occurs at a higher substrate concentration than normally required for the saturation of freely soluble enzyme and hence a higher $K_{m}$ value. A similar change was observed in pigeon pea urease immobilized on chitosan beads and jack bean urease immobilized in a polyacrylamide gel.

**Conclusions**

The study on the different covalent coupling methods on different matrices indicated that periodate mediated coupling to sepharose gel was most efficient. Nylon bead immobilized urease retained its activity even after 30 days. Immobilized urease retained its activity over a wider pH and temperature range compared to the free enzyme.
References