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Improved stability of urease upon coupling to alkylamine and arylamine glass and its analytical use

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Abstract

Biocatalyst stability is a major concern in almost all bioprocesses, because it may affect the overall cost of the process. Immobilization is a cost-effective approach and in the present work, pigeonpea urease was covalently coupled to alkylamine glass via glutaraldehyde activation and arylamine glass via diazotation. This coupling resulted in 92.5% and 90% immobilization, respectively. The immobilized urease showed optimum activity at 77 °C and retained 50% of its activity when incubated at this temperature for 90 min. This immobilized enzyme was quite stable at higher temperatures and over a broad pH range; unusually stable upon storing at 4 °C and also retained 50% activity even after 25 reuses. Hence, the obtained results are much better compared to other matrixes used so far for urease immobilization. This preparation was also successfully used in potentiometric biosensing for the estimation of blood urea from clinical patients. Hence, coupling of urease to glass via this mode of coupling could therefore be a versatile tool for immobilization of proteins and would have great promise in clinical as well as industrial use.

Keywords: Alkylamine; Arylamine; Glass; Immobilization; Pigeonpea (*Cajanus cajan*); Urease; Potentiometry; Stability

1. Introduction

Urease is found in a wide range of organisms, many have been isolated from various bacteria, higher plants, fungi and some invertebrates [1]; urease (urea amidohydrolase, EC 3.5.1.5) is a hexameric protein (540 kDa) made up of six identical subunits [2,3] and a nickel-dependent metalloenzyme catalyzes the hydrolysis of urea to ammonia and carbamate. The carbamate then spontaneously hydrolyzes to form carbonic acid and a second molecule of ammonia. At physiological pH, the carbonic acid proton dissociates and the ammonia molecules equilibrate with water becoming protonated, resulting in a net increase in pH:

$$(NH_2)_2CO + 3H_2O \xrightarrow{\text{Urease}} CO_2 + 2NH_4OH$$

Urease from pigeonpea (*Cajanus cajan*) was purified in our laboratory [3] and has certain advantages over jack bean urease (JBU) like: (1) the source, pigeonpea, is much cheaper than the jack bean seeds commonly used for the extraction of urease, (2) the pigeonpea urease (PPU) exhibits higher stability compared with the commonly used JBU [4], (3) the higher specific activity of immobilized PPU over JBU under similar conditions [5].

Medically, bacterial ureases are important virulence factors. In an agricultural circumstance, rapid hydrolysis of fertilizer urea by soil bacterial ureases results in unproductive volatilization of nitrogen and may cause ammonia toxicity or alkaline-induced plant damage. Lastly, the enzyme is important clinically in assaying for urea [6], since elevated levels of urea are clinically significant in many renal disorders such as uremia, kidney malfunctioning, etc.

Biocatalysts are inherently labile and required to perform in an environment quite different from its natural habitat and they seldom have the features adequate to be used as industrial catalysts. The biocatalyst stabilization under operation conditions is a key issue of biocatalysis and bioprocess technology; partly this problem can be solved by immobilization. Immobilization was actually developed in the 1950s and still there is a constant hunt for the best immobilization matrix. The attractions of immobilized enzymes from an analytical standpoint are primarily their reusability, and hence cost saving, also the greater efficiency and control of their catalytic activity (e.g., potentially longer half-lives, predictable decay rates and more efficient multi-step

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reactions). The pigeonpea urease was immobilized by adsorption onto inert solids, like flannel cloth [7], ion-exchange resins, like DEAE-Cellulose [8], or physically entrapped/encapsulated in solids, such as cross-linked gels like, polyacrylamide and calcium alginate [9], gelatin [4], chitosan [10], reverse micelles [11], microspheres [5] and hollow fibers.

The use of glass as an immobilization system has proven to be effective with a variety of enzymes and other proteins [12–14] and it has also been used for optical biosensing [15–17], as a stationary phase in chromatography [18], for protein chip construction [19], oligonucleotide microarrays [20,21] and protein–protein interaction studies [22]. Different bifunctional agents have been used in amine-cross-linking; glutaraldehyde has been most widely used and at the same time its mode of cross-linking is still an enigma. Many texts indicate that the dialdehyde makes linking imines between the matrix and the protein amine [23]. The arylamine can be converted to diazonium-Glass, which is capable of reacting with imidazoles and phenolic compounds, this coupling method was first used for enzyme immobilization by Weetall and Filbert [24]. The diazonium activated arylamine matrix was used in immobilization of 3α-hydroxysteroid dehydrogenase, diaphorase [25], bacterial luciferase and FMN reductase [12]. The diazonium reagents are also used to functionalize single-walled carbon nanotubes [26].

Operational stability (like thermal, pH and shelf-life) of the immobilized enzyme is a very important parameter in considering it for industrial use. In the last few years, improvements in carrier and immobilization techniques are opening new options for process development. Despite its great technological potential, few large-scale processes utilize immobilized enzymes, due to activity losses, diffusional restrictions and additional costs. These issues can be overcome with cheaper purification (as in the present case, urease from pigeonpea), high percent immobilization, which if it leads to improved stability.

In the present investigation, we have immobilized urease on alkylamine glass via glutaraldehyde cross-linking and arylamine glass via diazotation reaction by testing various conditions like cross-linker concentration, protein concentration, activation time and coupling time. The kinetics and stability parameters for both free and immobilized urease were studied in greater detail. The reusability and shelf life were also studied which are important criteria in judging the efficacy of the immobilization. Finally, this immobilized urease was attached to a combined pH electrode and used in potentiometric biosensing of urea in clinical blood samples. The immobilized enzyme offers the advantage of better pH, temperature and storage stability over free urease; can be used repeatedly and also offers promise in clinical estimation of urea.

1.1. Mode of binding

Alkylamine glass–urease:

$$\text{glass–R}\cdots\text{NH}_2 + \text{glutaraldehyde} \xrightarrow{27\,^\circ\text{C}} \text{glass–CHO} \xrightarrow{\text{urease–NH}_2} \text{glass–CH}_2\text{NH–urease}$$

Arylamine glass–urease:

$$\text{glass–Ar}\cdots\text{NH}_2 + \text{HCl, NaNO}_2 \xrightarrow{\text{ice}} \text{glassArN}_2\text{–Cl} \xrightarrow{\text{urease–NH}_2} \text{glass–ArN} \equiv \text{N–urease}$$

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Urease (urea amidohydrolase, EC 3.5.1.5) was isolated from dehusked pigeonpea seeds procured from the local market. Alkylamine and arylamine glass, glutaraldehyde and dialysis tubing were purchased from Sigma Chemical Co., St. Louis, MO, USA. Urea (enzyme grade), Tris were obtained from Sisco Research Laboratories and Nessler's reagent from HiMedia Laboratories, Mumbai, India. All other chemicals used were of fine grade. All solutions were prepared in Milli-Q (Millipore, USA) water.

2.1.2. Urease purification and activity measurements

Urease was purified according to Das et al. [3]. The enzyme used in the present study has a specific activity of 1250–1750 Units mg^{-1} protein (varied from batch to batch). All activity measurements were done in triplicate. One unit of urease activity liberates 1 μmol of NH_3 from urea per minute at pH 7.3 and 27 °C. The amount of NH_3 liberated on incubating the free and immobilized enzyme with 0.2 M urea in a fixed time period at an enzyme-saturating concentration of urea was determined using Nessler's reagent; the yellow–orange colour produced was measured spectrophotometrically at 405 nm (ATI-UNICAM UV–vis spectrophotometer, UK). The amount of NH_3 liberated in the test solution was calculated by calibrating the Nessler's reagent with standard NH_4Cl solution.

2.1.3. Protein estimation

The concentration of free enzyme was determined by Lowry et al. [27] and for the immobilized protein, we have adopted a modification of the method of Lowry to measure protein bound to alkyl and arylamine glass [28]. This procedure is unaffected by the amino compounds arising from the support. Copper reagent was prepared fresh daily by mixing 0.5 mL of 1% $CuSO_4{\cdot}5H_2O$, 0.5 mL of 2% Na–K tartarate, and 50 mL of 2% Na_2CO_3. In the standard Lowry method, the Na_2CO_3 is in 1.0N NaOH. Commercial Folin–Ciocalteu reagent was diluted to 1.0N in HCl prior to use. Samples of immobilized protein were incubated in 0.2 mL of 1.0N NaOH for 1 h at 30 °C. Copper reagent, 2.0 mL, was added, and the samples were incubated for 30 min at 30 °C. Folin–Ciocaleu reagent, 0.2 mL, was added, with mixing, and the samples were incubated for 30 min at 30 °C. The absorbance of the samples was then measured versus reagent blank at 750 nm in a spectrophotometer. For standards, BSA or urease were dissolved in 1.0N NaOH immediately before use.

2.2. Immobilization

2.2.1. Urease coupling to alkylamine glass

To each 10–50 mg of alkylamine glass was added 300 μL of 0.5–2.5% solution of glutaraldehyde ($C_5H_8O_2$) in 0.05 M Tris–acetate buffer, pH 7.0. The mixture was allowed to stand for different time intervals at room temperature. The glutaraldehyde activated glass was then washed with 0.05 M Tris–acetate buffer, pH 7.0 twice. Later, 0.1–10 mg enzyme was added to the beads and the coupling was continued for different time intervals at 4 °C. Then, the remaining (unbound) enzyme solution was removed and estimated for protein and activity. The urease-alkylamine glass beads were washed slowly with ice-cold Tris buffer, pH 7.0. In order to determine the optimum immobilization conditions, the following parameters during the immobilization process were studied: (a) amount of alkylamine glass: 5–20 mg, (b) glutaraldehyde concentration: 0.5–2.5% (w/v), (c) protein concentration: 0.01–10 mg, (d) activation time (glass + glutaraldehyde): 1–12 h, (e) coupling time (activated glass + urease): 1–24 h. The percentage immobilization is calculated by using the following formula:

$$\text{Percentage immobilization (\%)} = \frac{\text{Specific activity of immobilized urease (U/mg)}}{\text{Specific activity of free urease (U/mg)}} \times 100$$

2.2.2. Urease coupling to arylamine glass

Arylamine glass (10–50 mg) is first dissolved in 300 μL of varying concentrations of hydrochloric acid (0.1–5N), and then solid sodium nitrite (1–5 mg) is added and the reaction was carried out for 30 min in ice bath. The activated diazonium glass were then washed with 1 mL ice-cold 1% sulfamic acid twice, followed immediately with 1 mL ice-cold 0.05 M Tris–acetate buffer, pH 8.0, thrice. To the activated (brick red–orange) glass beads, enzyme (0.1–10 mg) was added and the reaction was continued for different time intervals at 4 °C. Then, the remaining (unbound) enzyme solution was removed and estimated for protein and activity. The urease-arylamine glass beads were washed slowly with ice-cold Tris buffer, pH 7.0. In order to determine the optimum immobilization conditions, the following parameters during the immobilization process were studied: (a) amount of arylamine glass: 5–20 mg, (b) HCl concentration: 0.1–5N, (c) sodium nitrite: 1–5 mg, (d) protein concentration: 0.01–10 mg, (e) coupling time (activated glass + urease): 1–24 h. The percentage immobilization was calculated as above.

2.3. Steady-state kinetics

2.3.1. Optimum pH

The optimum pH for the free, alkylamine and arylamine glass coupled ureases were studied in the pH range 5.0–9.0 at room temperature. The free and immobilized ureases were incubated in buffers 0.05 M sodium acetate for pH 5.0–6.0 and 0.05 M Tris–acetate for pH 6.5–9.0. The substrate urea (0.2 M), which is prepared in respective pH buffers, was added to the reaction mixture and the enzyme activity was measured, as stated earlier.

2.3.2. Optimum temperature

The optimum temperature for the free, alkylamine and arylamine coupled ureases were studied at temperatures between 27 and 97 °C in the respective optimum pH buffers (free: 7.3, alkyl: 6.8, and aryl: 7.0). The free and immobilized ureases were incubated at different temperatures and the pre-incubated substrate at respective temperatures was added to the reaction mixture and the enzyme activity was measured, as stated earlier.

2.3.3. K_M and V_{max}

The activity as a function of substrate concentration was measured for free and immobilized enzymes; the K_M (an approximate measure of the affinity of the substrate for the enzyme) and V_{max} (the maximal velocity when enzyme is saturated with the substrate) were determined by using Lineweaver–Burk plot with the help of SigmaPlot 9.0 software.

2.4. Stability

2.4.1. pH

The effect of pH on the stability of free and immobilized (alkyl and arylamine) urease was tested in the pH range 3.5–9.0. The buffers used were 0.05 M Sodium acetate for pH 3.5–6.0 and 0.05 M Tris–acetate for pH 6.5–9.0. The free and immobilized ureases were incubated in respective pH buffers for 12 h at room temperature. The residual urease activity was measured as mentioned earlier.

2.4.2. Thermal inactivation

The temperature stability (thermal inactivation) was studied by incubating the free and immobilized (alkyl and arylamine) urease at 77 °C for different time intervals; this was done by keeping different samples of each free, alkyl and arylamine ureases for different time intervals (0–4 h). The samples were taken out at different time intervals, chilled immediately in ice and allowed to come back to room temperature, after which the residual urease activity was measured.

2.4.3. Reusability

The immobilized (alkyl and arylamine) ureases were stored under semi-dry and wet conditions incubated at 4 °C and room temperature, respectively. In the case of semi-dry condition, the immobilized urease was stored without buffer and in the wet condition the immobilized urease was stored in the 0.05 M Tris–acetate for pH 7.0. The beads were reused for 10 times over a period of 8 days and the residual activity was measured. After the assay, the immobilized urease in dry condition was washed with buffer and the entire buffer was sucked out and was stored at 4 °C and room temperature, respectively. Further, the immobilized urease, which showed better stability, was reused for prolonged periods.

2.4.4. Storage

Several samples of immobilized (alkylamine and arylamine) and free ureases were stored in respective optimum pH buffers (0.05 M Tris–acetate for pH 6.8, 7.0 and 7.3) at 4 °C. The free and

immobilized ureases were tested for residual activity at regular intervals for over 70 days.

2.5. Potentiometric biosensing

The immobilized urease glass beads were used for the potentiometric biosensing of urea. The dialysis bag containing 50 mg of immobilized urease was tied with a thread at one end and the other end was tied to the sensing electrode of the pH meter. The potential response due to ammonia released is tested in 10 mL reaction volume, under varying conditions (a) water + 50 mM KCl, (b) 1 mM Tris + 50 mM KCl buffer, pH 7.0, (c) 10 mM Tris + 50 mM KCl buffer, pH 7.0. The electrode with the immobilized urease was allowed to stand in the solution for signal stability, after which 200 μL of known concentration (0–150 mg/dL) of urea was added to 10 mL reaction volume under constant stirring and the corresponding potential changes were monitored. In order to measure the urea content of serum samples obtained from S.S Hospital, BHU, Varanasi, India; 200 μL of serum was added to the reaction volume and the urea was estimated using the standard plot prepared with the known urea concentrations (see Section 3).

3. Results and discussion

3.1. Immobilization

3.1.1. Alkylamine- and arylamine-urease

The alkylamine glass appears transparent (Fig. 1A) and once activated with glutaraldehyde the glass appears orangish in colour (Fig. 1B). In case of alkylamine-urease immobilization, various conditions (Table 1) have been tested to obtain optimum immobilization and the best conditions found to be, 10 mg glass when activated with 1% glutaraldehyde for 6 h and when 1 mg of urease was coupled to the activated glass for 12–24 h had resulted in 92.5% immobilization.

Table 1
The various conditions tested and percentage immobilization obtained for alkylamine-urease coupling

Glutaraldehyde (%)	0.5	1.0	1.5	2.0	2.5	–
Immobilization (%)	89.6	92.5	89.0	86.0	82.0	–
Alkylamine glass (mg)	5	10	15	20	–	–
Immobilization (%)	87.5	92.5	86.5	85.0	–	–
Activation time (h)	1	2	6	12	–	–
Immobilization (%)	85.0	90.0	92.5	92.0	–	–
Urease (mg)	0.01	0.1	0.5	1	5.0	10.0
Immobilization (%)	90.0	90.0	92.0	92.5	90.0	85.0
Coupling time (h)	1	6	12	24	–	–
Immobilization (%)	85.0	90.5	92.0	92.0	–	–

Bifunctional agents like, glutaraldehyde is the most common cross-linking agent, which often reacts with the lysine amino groups of an enzyme and is used to cross-link protein with the matrix. Glutaraldehyde coupling is an enigma that deserves special attention as, it could either stabilize the enzyme, due to the multi-point attachment, presumed to occur with glutaraldehyde, prevents unfolding of the protein [29]. Alternatively, the polymeric nature of glutaraldehyde provides a long leash, attaching the protein to the matrix, which may permit greater flexibility for

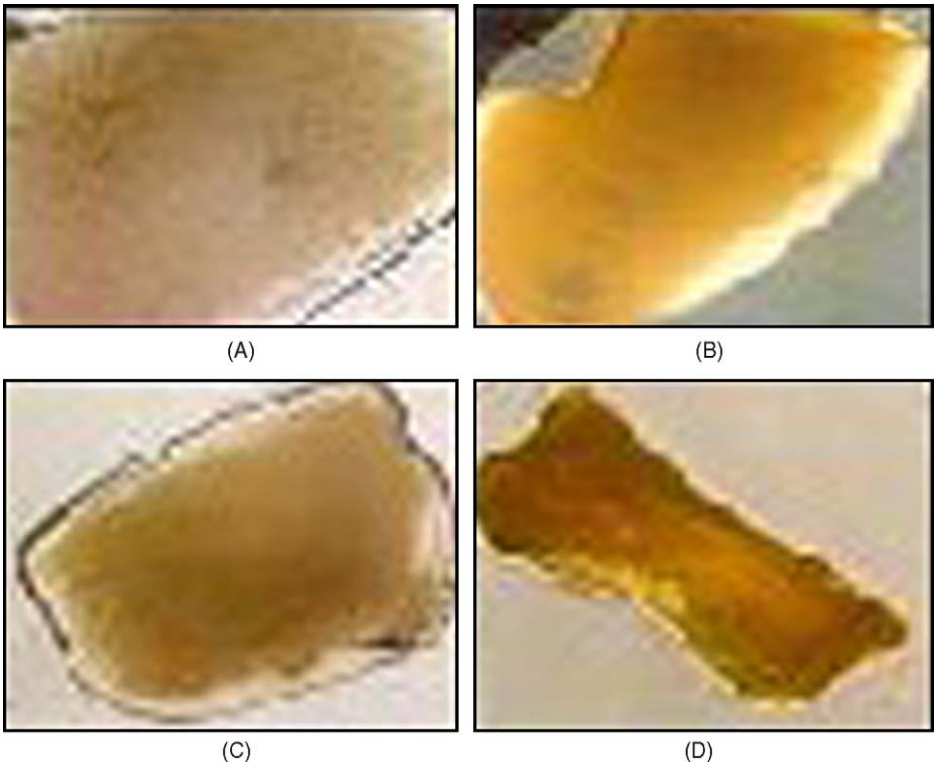

Fig. 1. Microscopic images of (A) plain alkylamine glass, (B) urease coupled to glutaraldehyde activated alkylamine glass, (C) plain arylamine glass, (D) urease coupled to activated diazo-arylamine glass; at 10 × 10 magnification using Leica Inverted Microscope.

Table 2
The various conditions tested and percentage immobilization obtained for arylamine-urease coupling

HCl (N)	0.5	1.0	1.5	2.0	2.5	–
Immobilization (%)	77.0	84.5	86.0	90.0	75.5	–
Arylamine glass (mg)	5	10	15	20	–	–
Immobilization (%)	88.0	90.0	87.0	85.5	–	–
Sodium Nitrite (mg)	1	2	3	4	5	–
Immobilization (%)	85.0	88.5	90.0	82.5	79.0	–
Urease (mg)	0.01	0.1	0.5	1.0	5.0	10.0
Immobilization (%)	89.0	89.0	90	89.5	86.0	83.5
Coupling time (h)	1	6	12	24	–	–
Immobilization (%)	82.5	87.5	90.0	90.0	–	–

conformational changes required for activity. Some times, due to excessive cross-linking may lead to aggregation, precipitation and loss of activity, distortion of the 3D enzyme structure [30]. In the present study, increasing the concentration of glutaraldehyde from 1% to 2.5% (v/v) resulted in the decreased urease activity and hence decrease in immobilization from 92.5% to 82%, which could be due to the reaction between glutaraldehyde and thiol (–SH) groups of urease, which are essential for the catalytic activity of urease. Similar effect was also observed in chitin–glutaraldehyde coupling [31]. The protein concentration with which the maximal immobilization obtained was 1 mg; whereas with 10 mg protein the immobilization was 80%, this decrease could be due to the fact that there were not sufficient number of binding sites for 10 mg of protein and also partly could be due to aggregation of urease at such a concentration. The variation in activation time did not had much impact on immobilization but the variation in coupling time had an impact; incubation time of 1 h between urease and activated glass resulted in low immobilization (85%) where as incubation for 6 h resulted in maximal immobilization and incubation for extended periods did neither improved nor decreased immobilization. Thus, the coupling with glutaraldehyde is the simplest method; however, it is critical to remove excess glutaraldehyde before adding protein for optimal immobilization.

Similarly, arylamine glass appears dull orange in colour (Fig. 1C) and once activated by diazotization reaction they appear brick-red in colour (Fig. 1D). The arylamine-urease immobilization was tested under various conditions (Table 2). The reaction (activation) of arylamine glass in the presence of HCl and $NaNO_2$ in ice bath is crucial in obtaining the optimal immobilization. Arylamine reacts with nitrous acid differently depending on the temperature; due to this reason the reaction was carried out in ice bath. The activated diazonium salts are very unstable and yield carbocation-derived products by loss of the very good leaving group, N_2. Pre-treatment of arylamine glass with 2N HCl and 3 mg (solid) $NaNO_2$ had a maximum of 90% immobilization. The concentrations of HCl lower than 2N were not sufficient enough to activate the glass and the concentrations above had resulted in eroding the reactive sites of glass. In the case of $NaNO_2$, the concentrations beyond 3 mg resulted in a faster and explosive effervescence (as a result the Eppendorf tubes cap opened) and that had resulted in loss of beads and hence immobilization. The variation in coupling time between urease and activated arylamine glass had similar kind of results as observed in the case of alkylamine glass.

Urease, especially pigeonpea urease has been immobilized on several matrices like polyacrylamide and sodium alginate: 50% [9]; reverse micelle: >80% [11]; flannel cloth via polyethylenimine: 56% [7]; chitosan via glutaraldehyde: 64% [10]; DEAE-Cellulose paper: 51% [8]; gelatin: 75% [4]. The immobilization achieved for jack bean urease with different methods are: encapsulation in poly (methylene co-guanidine) coated alginate: 31% [32]; covalent binding to chitosan-poly(glycidyl methacrylate) copolymer: 82.15% [33]; aminated butyl acrylate ethylenedimethacrylate: 56% [34]; acrylonitrile copolymer: 68% [35]; composite hydrogel: 55% [36]. Hence, the immobilization achieved with alkylamine via glutaraldehyde cross-linking for pigeonpea urease was much higher than the methods used so far. Apart from immobilization, the alkyl and arylamine glass has several advantages over soft gels, especially in processes that require a fast flow rate or high pressure because the flow rates with glass are linear with pressure. The mechanical strength of glass could provide reproducible results with constant column parameters. Also, this matrix is resistant to biological degradation and compatible with almost all organic solvents and most concentrated acids. Where as in some instances like stirring, soft supports may be advantageous as compared to more rigid supports.

3.2. Enzyme kinetics

3.2.1. Optimum pH

The optimum pHs for free and immobilized urease (alkylamine and arylamine) are shown in Fig. 2. The optimum pH was found to be 7.3, 6.8 and 7.0, respectively for free, alkylamine- and arylamine-ureases. Similar results were obtained recently on DEAE-Cellulose immobilization [8]. It could be interpreted that there is a little shift of the optimum pH upon enzyme immobilization. Different pH-activity profiles of immobilized urease

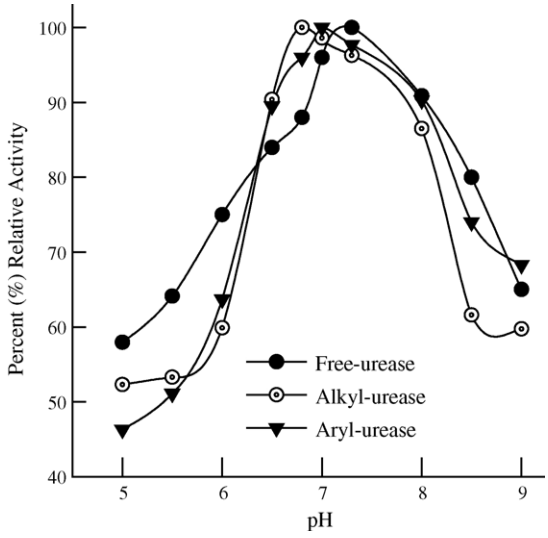

Fig. 2. Optimum pH for the activity of free, alkylamine- and arylamine-urease.

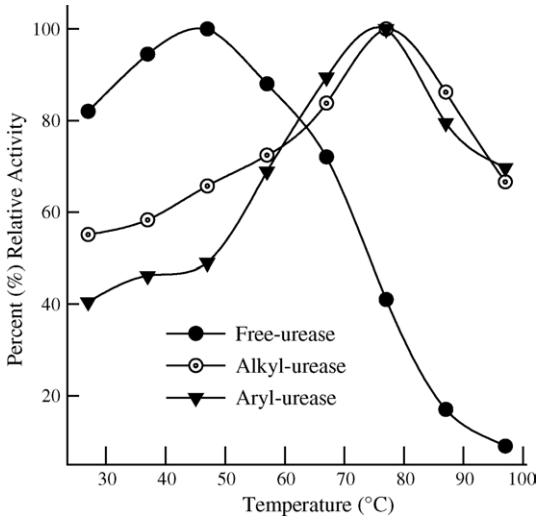

Fig. 3. Optimum temperature for the activity of free, alkylamine- and arylamine-urease.

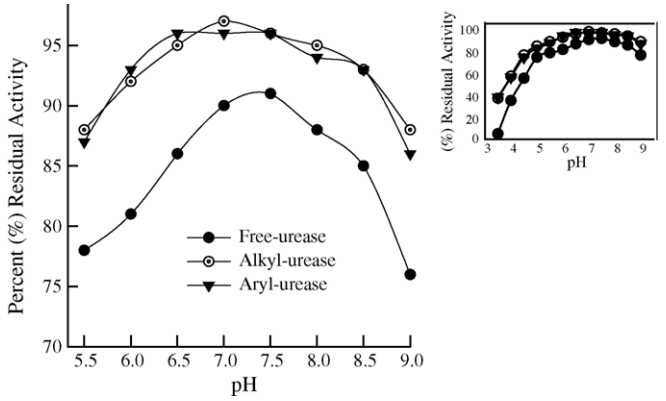

Fig. 4. pH stability of free, alkylamine- and arylamine-urease in the range 5.5–9.0 (expanded scale) and the inset shows a (complete scale) in the range 3.5–9.0.

have been obtained depending on the support chosen and immobilization method adopted [37].

3.2.2. Optimum temperature

The optimum temperature for the free urease was 47 °C; whereas the optimum for both, the urease coupled to the alkylamine and arylamine glass was 77 °C (Fig. 3); the immobilized urease was more active at high temperatures as it resisted denaturation. It could be due to the fact that under normal conditions in living cells, most of the enzymes are either bound to membrane or to other macromolecules and rarely exists in free form as in vitro experiments. This similar displacement of optimum temperature for immobilized enzymes was observed in many cases, but the extent of displacement would differ from matrix to matrix and the kind of interaction between the enzyme and matrix. The displacement of optimum temperature from 45 to 65 °C was observed with DEAE-Cellulose and gelatin bound pigeonpea urease [8,4]. The similar shift was observed in case of immobilized jack bean urease [34,38,39].

3.2.3. K_M and V_{max}

The kinetic parameters K_M and V_{max} for the free and immobilized urease were determined by measuring the rate of urea hydrolysis as a function of substrate concentration, keeping constant the units of urease activity utilized in the assays. The K_M and V_{max} for free urease are 3.35 mM and 1.39×10^3 μmole $NH_3\,min^{-1}\,mg^{-1}$ protein, and that for alkylamine-urease are 3.55 mM and 1.33×10^3 μmole $NH_3\,min^{-1}\,mg^{-1}$ and for arylamine-urease are 3.43 mM and 1.39×10^3 μmole $NH_3\,min^{-1}\,mg^{-1}$ protein. The invariance of K_M suggests that there was no interaction between urea and glass matrix, so that the concentration of substrate necessary for the maximum reaction rate to occur is the same for free and immobilized urease and also suggests that there are no changes in the accessibility of substrate to the active site as there are no diffusional resistances of stagnant solvent layers produced around the immobilized molecules. Others [40,41] also have observed the unchanged K_M values upon immobilization. The significant changes (increase) in K_M upon immobilization could be observed most commonly in case of encapsulation and adsorption methods viz., a 1.5–5 times higher K_M has been observed [4,8,10,34]. The insignificant change of V_{max} could be due to fact that the conformational changes in tertiary structure of urease and steric effects resulting from limitation of the accessibility of substrate to the active site are unaffected on immobilization and hence no reduction in catalytic efficiency [42]. No significant changes in V_{max} upon immobilization have been observed with different ureases [8,43]. As there were no significant changes in either K_M or V_{max} upon immobilization of urease to alkylamine and arylamine glass implies that this matrix would not pose problems arise due to diffusional constraints in bioreactor and flow injection process.

3.3. Stability

3.3.1. pH

The free urease when incubated in buffer, pH 3.5 at room temperature for 12 h retained only 5% of its original activity, where as the alkylamine and arylamine-urease retained 37% and 38%, respectively (Fig. 4 and inset). There was a significant increase in the activity of immobilized urease at low pH values as compared to the free enzyme and has also a broad pH range, which is clearly shown in Fig. 4. This increased stability is due to the protection afforded by the matrix and additional stabilizing bonds between enzyme-matrix, which needed higher energy to break and so higher pH. The similar increased stability of immobilized (*Bacillus pasteurii*) urease, at low pH was also observed by Ciurli et al. [43].

3.3.2. Temperature

The thermal stability of free and immobilized-urease was investigated 77 °C (Fig. 5). After 15 min of incubation, the free urease had a residual activity of 22%, whereas alkyl and arylamine-urease had 96 and 98%, respectively. After 30 min incubation, free urease lost all of its activity, due to irreversible denaturation due to covalent changes such as the deamination of asparagine residues or non-covalent changes such as the rear-

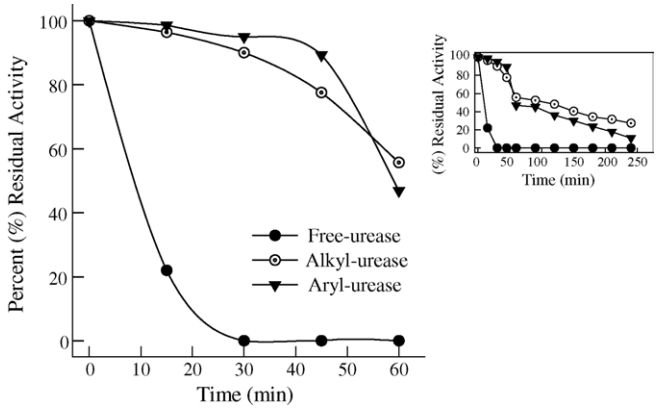

Fig. 5. Temperature inactivation at 77 °C of free, alkylamine- and arylamine-urease for 60 min (expanded scale) and the inset shows a (complete scale) up till 240 min.

rangement of the protein chain. The elevated temperatures can cause a change in the tertiary structure of a protein (mainly by breaking H-bonds). Even after 90 min of incubation immobilized urease retained 50% of activity. It is clear that the thermal stability was significantly improved upon coupling of urease to glass. In the present case, it could also be that the cross-linking limits the thermal movement of the molecules and greater stress, which if is not uniformly distributed along the protein chains, is accumulated in the polypeptide structure, leading to its disruption. Thermal stability upon immobilization is the result of molecular rigidity and the creation of a protected microenvironment. This improved thermo stability might be useful in the application of this system at high temperatures, avoiding the microbial contamination as well as the solubility of substrate and products is higher; which would have good scope in industrial use.

3.3.3. Reusability

The alkylamine-urease in semi-dry condition stored at 27 °C showed a residual activity of 7% after 10 reuses and when stored at 4 °C showed 27%; whereas in wet condition stored at 27 and 4 °C after 10 reuses showed 30% and 90% residual activity, respectively (Fig. 6A). In case of arylamine-urease in semi-dry condition stored at 27 and 4 °C showed a residual activity of 6% and 15%, respectively; where as in wet condition stored at 27 and 4 °C showed 18% and 62%, respectively (Fig. 6B). Hence, the immobilized enzyme is more stable in wet condition than in semi-dry condition and storing it at 4 °C is more favourable over 27 °C. There is a tremendous decrease in activity when the immobilized urease was not stored in buffer (i.e. semi-dry condition) and storing it at 4 °C improved the longevity (stability). Then both the alkyl and arylamine-urease in wet condition stored at 4 °C were further reused (30 times) and the residual activity are 40% and 20%, respectively (Fig. 6C). This result also implies that the strength of binding between urease and alkylamine via glutaraldehyde cross-linking would have been more than arylamine glass, as the activity after reusability was more for alkylamine. The activity loss upon reuse could be due to weakening in the strength of binding between the matrix and enzyme, these interactions might weaken on repeated use and

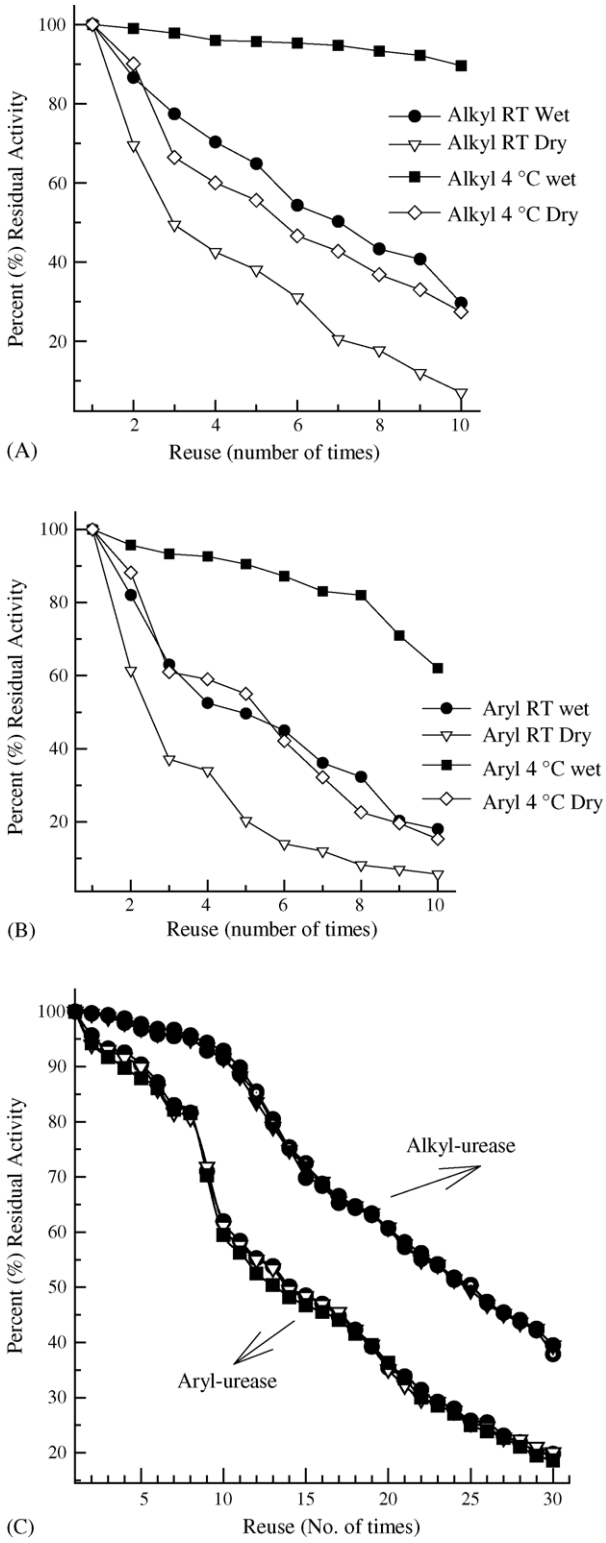

Fig. 6. (A) Reusability of alkylamine-urease under semi-dry and wet conditions at RT (room temperature) and 4 °C. (B) Reusability of arylamine-urease under semi-dry and wet conditions at RT (room temperature) and 4 °C. (C) Prolonged reusability of alkylamine and arylamine-urease under wet conditions at 4 °C, the values are plotted in triplicates.

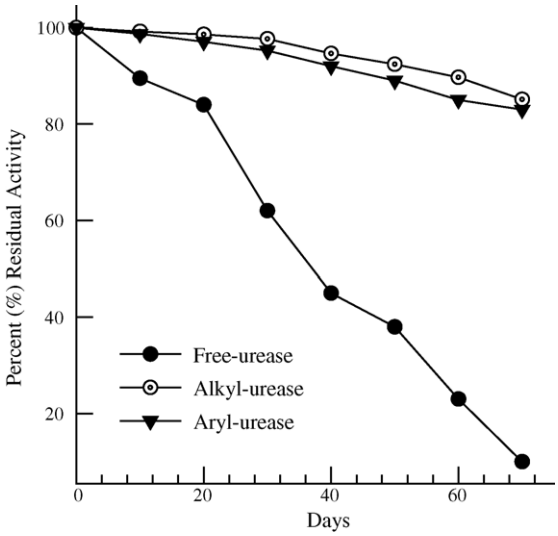

Fig. 7. Storage stability at 4 °C of free, alkylamine- and arylamine-urease for 70 days.

hence the enzyme might detach from the matrix and so a loss in activity; another reason could be that the frequent encountering of substrate into the same active site might distort it and this distortion would dwindle the catalytic efficiency either partially or fully. Immobilization of urease on Vermiculite using glutaraldehyde resulted in 61% residual activity after four repeated uses [44a] and 65% after five reuses [44b]; immobilization on aminated butyl acrylate ethylene dimethacrylate copolymer resulted in 50% residual activity after seven reuses [34]; on chitosan membrane resulted in complete loss after nine reuses [38].

3.3.4. Storage

The loss of activity, for free urease after 70 days of storage at 4 °C was 90% and in comparison to alkylamine- and arylamine-urease the losses were only 15% and 17%, respectively (Fig. 7). This clearly signifies that coupling of enzyme to glass matrix had tremendously improved the storage stability of urease and this is one of the biggest advantages of urease immobilization on glass. In case of DEAE-Cellulose immobilized urease the half-life was 150 days at 4 °C [8]; gelatin-immobilized urease was 240 days [4]; chitosan beads was 110 days [10]; alginate beads was 75 days [9] and flannel cloth was 70 days [7]. High storage stability was also reported in case of aminated butyl acrylate ethylenedimethacrylate copolymer with almost no loss of activity for 85 days [34]; 38% and 27% loss in case of Amberlite MB-1 [44b] and Chitosan-poly (GMA) Copolymer [33] at 4 °C after 60 days, respectively. Urease immobilized on modified polysulphone membrane had 73% residual activity after 30 days [45]. The urease immobilization on glass showed better shelf-life than many matrices used so far.

3.4. Potentiometric biosensing

The potential response due to NH_3 as a result of urease action on urea in (a) Milli-Q water + 50 mM KCl; (b) 1 mM Tris + 50 mM KCl buffer pH 7.0; (c) 10 mM Tris + 50 mM KCl buffer pH 7.0 were shown in Fig. 8A. The potential sensitivity of the biosensor, denoted S, can be calculated as: $S = E(+\infty) - E(-\infty) = A1 - A2$; where $A1$: initial E value = $E(+\infty)$ and $A2$: final E value = $E(-\infty)$. The S with Millipore water = 189 mV, 1 mM buffer = 110 mV, 10 mM buffer = 64 mV. The sensitivity is high when the reaction was carried out in Milli-Q water, but the response reaches saturation at 110 mg/dL; in case of reaction carried out in 1 mM buffer, the sensitivity was high and response reached saturation beyond 150 mg/dL. The reaction carried out in 10 mM buffer has a low sensitivity and there were no potential changes till 40 mg/dL because the buffer resisted the early changes in the pH and urea concentration of 40 mg/dL is a useful concentration clinically. Hence, we have chosen to carry out the potentiometric measurements in 1 mM buffer + 50 mM KCl.

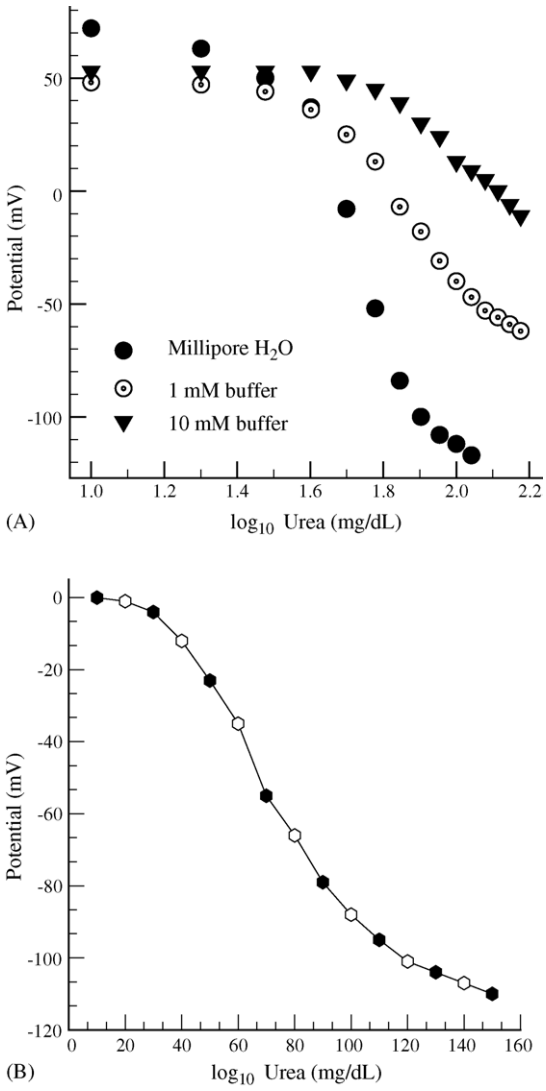

Fig. 8. (A) Calibration plot with known concentration of urea in reaction volume of (i) Millipore water + 50 mM KCl, (ii)1 mM Tris + 50 mM KCl pH 7.0, (iii) 10 mM + 50 mM KCl pH 7.0. (B) Standard plot with known urea concentration in 1 mM buffer.

The calibration plot is shown in Fig. 8B. Earlier, similar setup was used for gelatin-immobilized urease [4] and has obtained a potential sensitivity of 70 mV with 0.025 mM buffer. In the

Table 3
Estimation of urea in serum using immobilized urease by potentiometric biosensing and a comparison with autoanalyzer®

Serum sample	Immobilized urease by potentiometric biosensing (mg/dL)	Autoanalyzer® (mg/dL)
1	42 ± 0.7	43
2	28 ± 0.2	28
3	15 ± 1.0	17
4	61 ± 0.5	62
5	29 ± 0.2	29
6	29 ± 0.2	29
7	82 ± 1.7	84
8	19 ± 0.8	20
9	36 ± 0.3	36
10	30 ± 0.3	31

present study, we have obtained a higher sensitivity and also the response time for each new addition was about 90–120 s. The serum samples were tested for urea levels with this setup and the values obtained (Table 3) were comparable to the values from standard autoanalyzer®. This immobilization method would invariably have lot of promise in Biosensor applications for clinical use.

4. Conclusions

Alkylamine and arylamine glass would be one of the best supports for urease immobilization due to its higher surface area, high immobilization, high mechanical, chemical stability; ease in manufacturing and long working life. Coupling of urease to the glass had tremendously improved the pH, temperature, storage stability and also improved reusability; also this preparation was successfully used for urea biosensing with high sensitivity. This immobilization would found important applications in clinical chemistry, bioprocess, and protein microarrays.

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