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Development of Potentiometric Urea Biosensor Based on *Canavalia ensiformis* Urease

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1. Introduction

The increasing number of potentially harmful pollutants in the environment calls for fast and cost-effective analytical techniques to be used in extensive monitoring programs. Additionally, over the last few years, a growing number of initiatives and legislative actions for environmental pollution control have been adopted in parallel with increasing scientific and social concern in this area (Rogers & Gerlach, 1996; Rodriguez-Mozaz et al., 2004; Rodriguez-Mozaz et al., 2005; Rogers, 2006). Nitrogen compounds are pollutant found in several industrial effluents, being its determination of extreme environmental importance.

Several methods are used to urea determination, including spectrophotometry, fluorimetry, potentiometry and amperometry. But some of these require a pretreatment or are unsuitable for monitoring in situ. For this reason there has been growing interest in the development of biosensors for these determinations.

The requirements for application of most traditional analytical methods to environmental pollutants analysis, often constitute an important impediment for their application on a regular basis. The need for disposable systems or tools for environmental applications, in particular for environmental monitoring, has encouraged the development of new technologies and more suitable methodologies. In this context, biosensors appear as a suitable alternative or as a complementary analytical tool. Biosensors can be considered as a subgroup of chemical sensors in which a biological mechanism is used for analyte detection (Rogers & Gerlach, 1996; Rodriguez-Mozaz et al., 2005; Rogers, 2006).

A biosensor (Figure 1) is defined by the International Union of Pure and Applied Chemistry (IUPAC) as a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in contact direct spatial with a transduction element (Thévenot et al., 1999). Biosensing systems and methods are being developed as suitable tools for different applications, including bioprocess control, food quality control, agriculture, environment, military and in particular, for medical applications. The main classes of bioreceptor elements that are applied in environmental

analysis are whole cells of microorganisms, enzymes, antibodies and DNA. Additionally, in the most of the biosensors described in the literature for environmental applications electrochemical transducers are used (Thévenot et al., 1999).
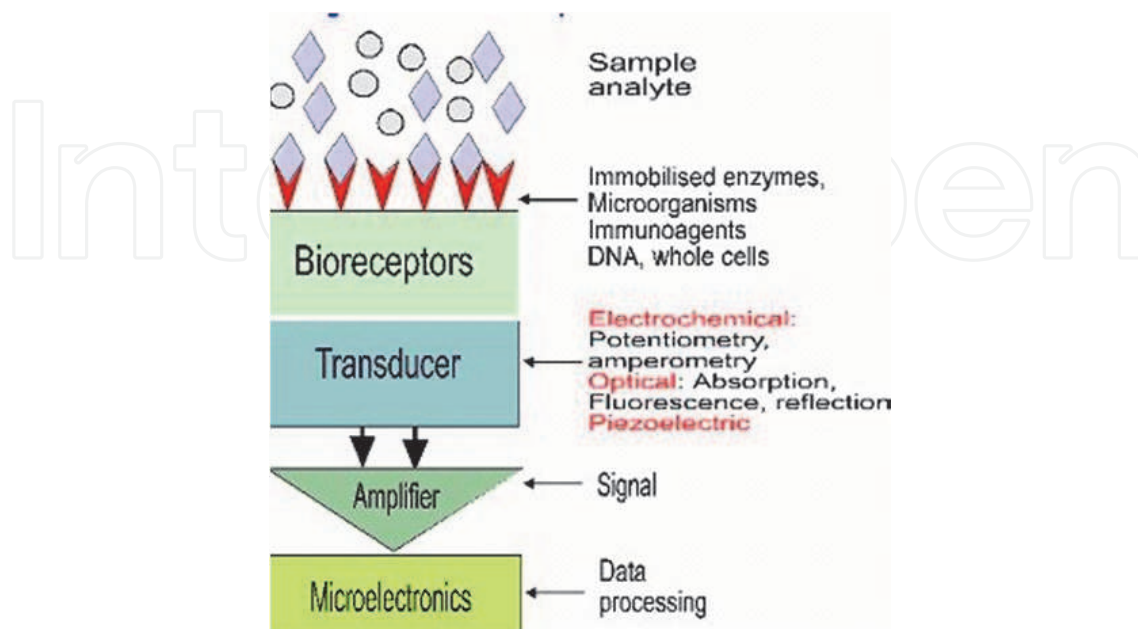

Fig. 1. Biosensor scheme.

For environmental applications, the main advantages offered by biosensors over conventional analytical techniques are the possibility of portability, miniaturization, work on-site, and the ability to measure pollutants in complex matrices with minimal sample preparation. Although many of the developed systems cannot compete yet with conventional analytical methods in terms of accuracy and reproducibility, they can be used by regulatory authorities and by industry to provide enough information for routine testing and screening of samples (Rogers & Gerlach, 1996; Rogers, 2006; Sharpe, 2003). Biosensors can be used as environmental quality monitoring tools in the assessment of biological/ecological quality or for the chemical monitoring of both inorganic and organic priority pollutants.

Due to great variety of vegetal tissues Brazil constitutes an inexhaustible enzyme source, which can be used in the most diverse areas of the knowledge, amongst them in the development of the biosensors. James B. Sumner (Sumner, 1926) crystallized the enzyme urease from jack bean, *Canavalia ensiformis* (Fabaceae), a bushy annual tropical american legume grown mainly for forage, in 1926, to show the first time ever that enzymes can be crystallized. Urease is abundant enzyme in plants and, moreover, it can be found at numerous of eukaryotic microorganisms and bacteria. The bacterial and plant ureases have high sequence similarity, suggesting that they have similar three-dimensional structures and a conserved catalytic mechanism.

Ureases (urea amidohydrolase, EC3.5.1.5) catalyzes the hydrolysis of urea to yield ammonia (NH_3) and carbamat, the latter compound decomposes spontausly to generate a second molecule of ammonia and carbon dioxide (CO_2) (Takishima et al., 1988) (Figure 2).

So, the main objective of this study was to optimize the operating conditions to obtain the final configuration of the urease biosensor for environmental application.
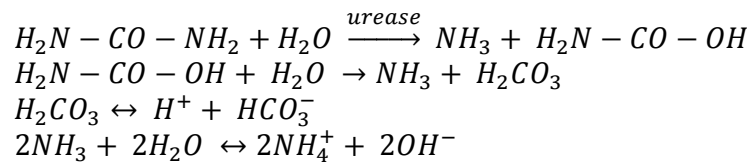
$$H_2N - CO - NH_2 + H_2O \xrightarrow{urease} NH_3 + H_2N - CO - OH$$
$$H_2N - CO - OH + H_2O \rightarrow NH_3 + H_2CO_3$$
$$H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$
$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^-$$

Fig. 2. Urea hydrolysis catalyzed by urease.

2. Material and methods

2.1 Biocomponent: jack beans (*Canavalia ensiformis*)

The biocomponent, jack beans, Canavalia ensiformis, as show in Figure 3, was donated by Seeds & Associate Producers on Earth by Brazilian Agricultural Research Company (EMBRAPA). It is a vegetal plant tissue rich in the urease (Luca & Reis, 2001). The jack beans were being used as a powder, with a particle size less than or 3mm, in free form or immobilized. When the powder was not in use, it was stored in refrigerators, till further use.

Fig. 3. Jack beans.

2.2 Ammonium ion-selective electrode calibration

For biosensor system development, an ammonium ion-selective electrode (*Orion Ammonia Electrode* 95-12 Thermo) was used as transducer. A calibration curve of the electrode potential (mV) vs. urea concentration (ppm) is constructed, using ammonium chloride solution (NH₄Cl) (1000 ppm) as stock solution. The standard solutions were prepared from the stock solution in range of 5 to 1000 ppm.

2.4 Best conditions of the fresh jack bean urease

The tests for optimization the enzymatic reaction conditions of fresh urease of jack beans monitored the urea hydrolysis to ammonia by ion-selective electrode response under different conditions. The conditions tested were: the jack bean amount (0.1, 0.2, 0.3, 0.4 and 0.5 g); the pH of sample standard solution (6.0, 7.0 and 8.0) and reaction temperature (20, 25, 30 and 40°C).

The assay consisted in adding the desired amount of powder in 5.0 mL of the standard solutions (several urea concentrations prepared from stock solution in potassium phosphate buffer with desired pH) and 100 µL of ISA (ionic strength adjustor buffer solution). Then the ammonium ion-selective electrode was immersed in the solution, monitoring the enzymatic reaction by the potential difference (mV) caused by urea hydrolysis.

2.5 Urease immobilization

The enzyme (powdered jack bean) immobilization using glutaraldehyde was performed according Junior (1995). **The final configuration of procedure, i**n brief, urease was covalently immobilized on nylon screen according to the following procedure: 0.2 g of powdered beans was placed under a nylon screen and 200 mL of glutaraldehyde solution (12.5%) were added. Then, another nylon screen was placed on top (Figure 4). After 20 minutes, the set was immersed in distilled water for 20 minutes and then in potassium phosphate buffer pH 7.0 at the same time. The immobilized biocomponent was used after storage for 24 hours in the refrigerator, at 4°C.
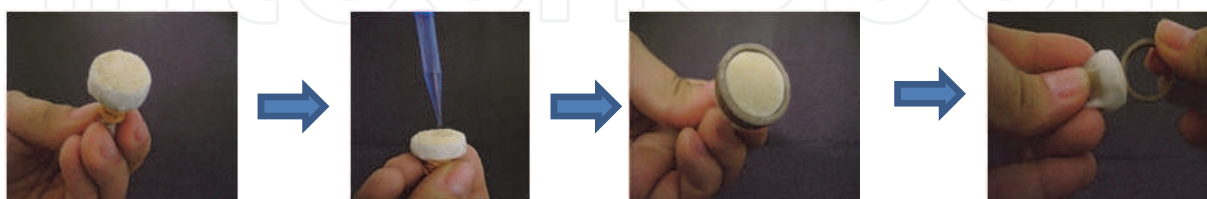
Fig. 4. Procedure step sequence of powdered jack bean immobilization.

2.6 Urease activity assay

Alkalimetric method is based on the observation made by Kistiakowsky & Shaw (1953, as cited in Comerlato, 1995) which the initial pH neutral of unbuffered solution of urea-urease rapidly increases to pH 9.0, and then remains approximately constant. The reaction products in this pH are usually ammonium carbamate, ammonium carbonate and bicarbonate as shown in the following Figure 5:

$$H_2NCONH_2 + 2H_2O \rightarrow (NH_4)^+ + (NH_2CO_2)^- + H_2O$$

$$\rightarrow 2(NH_4)^+ + (CO_3)^-$$

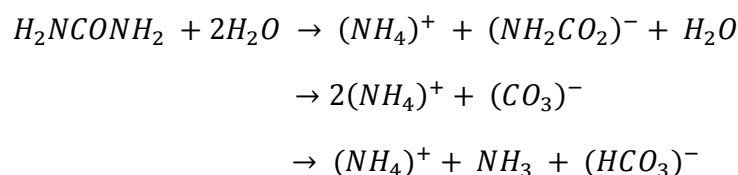$$\rightarrow (NH_4)^+ + NH_3 + (HCO_3)^-$$

Fig. 5. Urea hydrolysis by urease.

In this method, the urease activity was assayed by adding 1mL of urea solution, immobilized urease and 10mL of deionised water. Incubation was carried out at 25°C (room temperature) and low agitation for a constant interval. Withdrew an aliquot (2 mL) of mixture solution and terminated with hydrochloric acid solution. Then, the reaction mixture was back-titrated with sodium hydroxide solution, methylorange being used as an indicator. The blank test was assayed under the same conditions above, using 1mL of urea solution and 11 mL of deionised water.

These end products of the reaction are a buffer system that maintains the pH constant as the reaction proceeds. So using the substrate initially buffered at pH 9.0, avoids the subsequent change in pH. The addition of excess hydrochloric acid in the final time disrupts the reaction and converts the carbamate and ammonia to ammonium ions. Therefore, back-titration with sodium hydroxide measures the acid did not react (Comerlato, 1995).

To calculate the enzyme activity, first is necessary to calculate the volume of sodium hydroxide (vol. NaOH) wich is given by: *vol. NaOH = vol. NaOH blank – vol. NaOH test*. So the urease activity calculated using the equation below:

$$\frac{(NaOH\ molarity)x(vol.NaOH)x(1000)x(df)}{time\ reaction\ (minutes)x\ amount\ of\ jack\ beans\ (g)} = Units\Big/ amount\ of\ jack\ beans\ (g)$$

where:

$$vol.NaOH$$
$$= Volume\ (in\ milliliters)of\ sodium\ hydroxide\ solution\ used\ in\ the\ back\ titration$$

$$1000 = Conversion\ factor\ from\ millimoles\ to\ micromoles$$

$$df = Dilution\ factor$$

2.7 Kinetics parameters of urease

The kinetic parameters (K_m and V_{max}) for free and immobilized urease were determined by using Lineweaver–Burk plot. The substrate was urea, and its concentrations were 0.05 to 10.00% (w/v). The reaction rates were determined according to the method mentioned above in Section 2.4, with the established best reaction conditions. Based on Lineweaver–Burk plot Michaelis constant and maximal rate were calculated.

2.8 Instrumentation: biosensor system

The schematic set-up for biosensor system for urea analysis is presented as Figure 6. The set up consists of a peristaltic pump (2), reaction chamber (3) made from PVC pipe with biocomponent (immobilized urease) (4), transducer (ion-selective electrode) (5), potentiostat and data recorder (6). Standard sample and discard sample are numbered in Figure 5 as 1 and 7, respectively. Silicone tubing was used for connections.
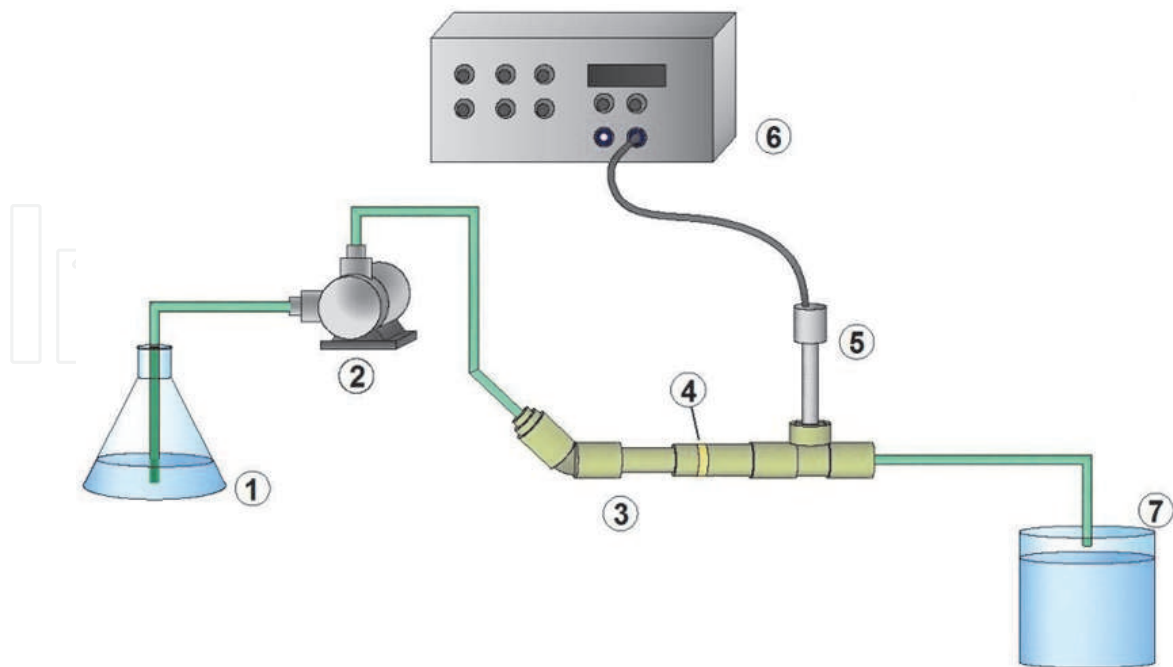

Fig. 6. Schematic set-up for biosensor system for urea analysis.

2.8.1 Procedure

For urea analysis, calibration standards were prepared by dilution of urea stock solution in potassium phosphate buffer, pH 6.0. All measurements were carried out by injection of 25.00 mL standard sample (0.50 a 50.00 ppm) at a flow rate of 40.00 mL.min⁻¹. After the sample has completed the reaction chamber, the pump was turned off and 200 mL of ISA was added and electrode was immersed. Then, data were collected throughout the reaction time, in order to analyze the response time of instrument. After each sample analysis, the system was thoroughly rinsed with distilled water for 2 minutes. The potentiometric measurements were made at room temperature (25°C).

A corresponding change of potential against the urea concentration could be observed. Different urea concentrations would cause different potential changes, due to ammonia generation. The values (mV) found with the transducer were converted into ammonia concentration through the equation of calibration curve of ammonium ion-selective electrode (Section 2.2). Thereby, the calibration curve of urea concentration versus ammonium generated was obtained.

2.9 Stability studies
2.9.1 Reusability

The immobilized urease was tested for its reusability by checking the biosensor response using assay as described in Section 2.8.1 at time intervals (days). After every use, biocomponent was washed properly with distilled water and stored in potassium phosphate buffer, pH 7.0 at 4°C, till further use.

2.9.2 Storage stability

The immobilized urease was stored in potassium phosphate buffer, pH 7.0 at 4°C. The activity was determined and recorded at regular intervals for stored urease using assay procedures described in Section 2.6. The values of activity were plotted against the number of days.

2.10 Protein assay

The amount of protein in the wash solutions after urease immobilization and biosensor system procedure were determined as described by Bradford (1976) with bovine serum albumin (BSA) as a standard.

2.11 Reproducibility

The reproducibility of ammonium ion-selective electrode response was checked by measuring this response when it was inserted into a 2% (w/v) urea solution with jack bean immobilized under over 2 minutes of enzymatic reaction. The assay was developed in potassium phosphate buffer, pH 6.0 at 25°C.

3. Results

3.1 Best conditions of the jack bean urease (fresh and immobilized)

Table 1 shows the values of ammonia concentration (ppm) generated by urea hydrolysis in 2 minutes of enzymatic reaction in experiments with several fresh jack beans weight with urea solutions of 0.05% to 10.00% (w/v).

Ammonia concentration (ppm)					
Urea concentration % (w/v)	Jack beans weight (g)				
	0.1	0.2	0.3	0.4	0.5
0.05	33.02	38.69	22.40	5.57	11.67
0.10	3.73	60.97	37.73	52.24	42.25
0.50	21.97	96.29	77.79	77.23	120.85
1.00	67.44	107.99	161.70	161.48	201.22
2.00	76.16	119.96	141.96	170.66	167.56
4.00	100.88	183.08	204.64	313.02	265.61
6.00	90.11	134.24	218.96	218.86	234.95
8.00	77.73	120.84	214.76	1690.26	157.23
10.00	48.09	104.86	130.99	157.26	195.89

Table 1. Results of experiments to choose the best jack beans amount analyzing the ammonia generation according Secion 2.4.

The table data are shown in the graph below (Figure 7). The curves of Figure 7 show that after the urea concentration of 4% (w/v) regardless of the jack beans amount was a saturation of the enzymatic reaction.
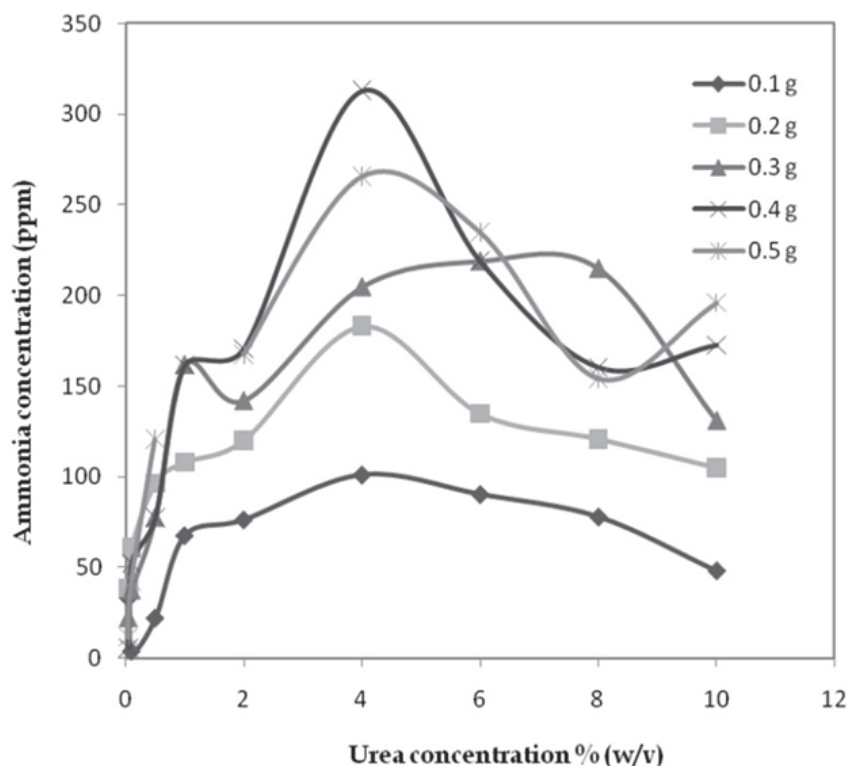
Fig. 7. Influence of jack beans amount on enzymatic reaction monitored (ammonia generation) by ammonium ion-selective electrode according Secion 2.4.

Figure 8 shows the pH dependence of buffer solutions on the potentiometric response of the transducer of fabricated urea biosensor. In the present work, the best response could be observed at pH 6.0 which was subsequently utilized in further experimental investigations.
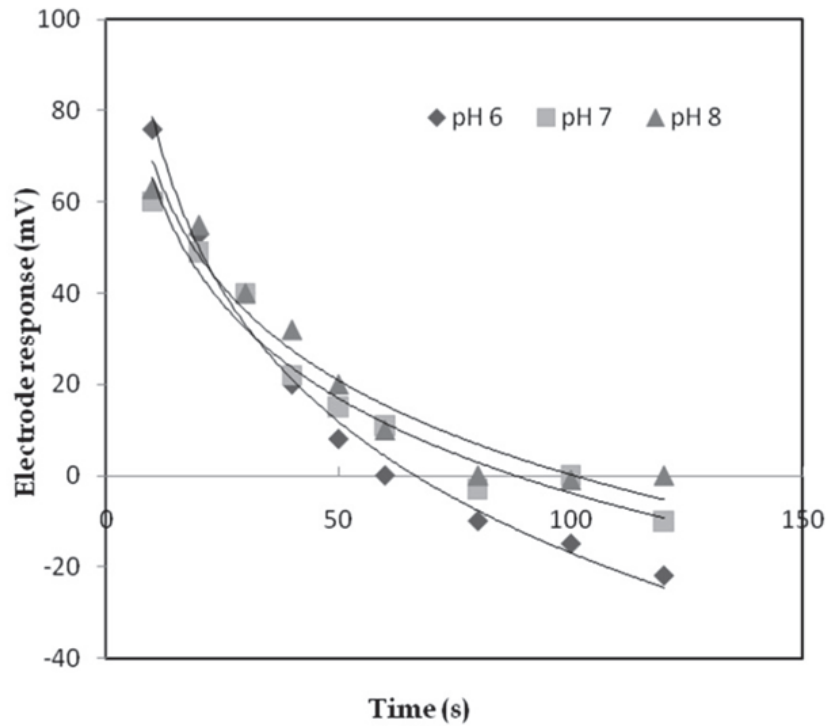

Fig. 8. Influence of buffer solution pH on the urea hydrolysis. Variation along the time of the ammonium ion-selective electrode (mV) response to a 2% (w/v) urea solution.
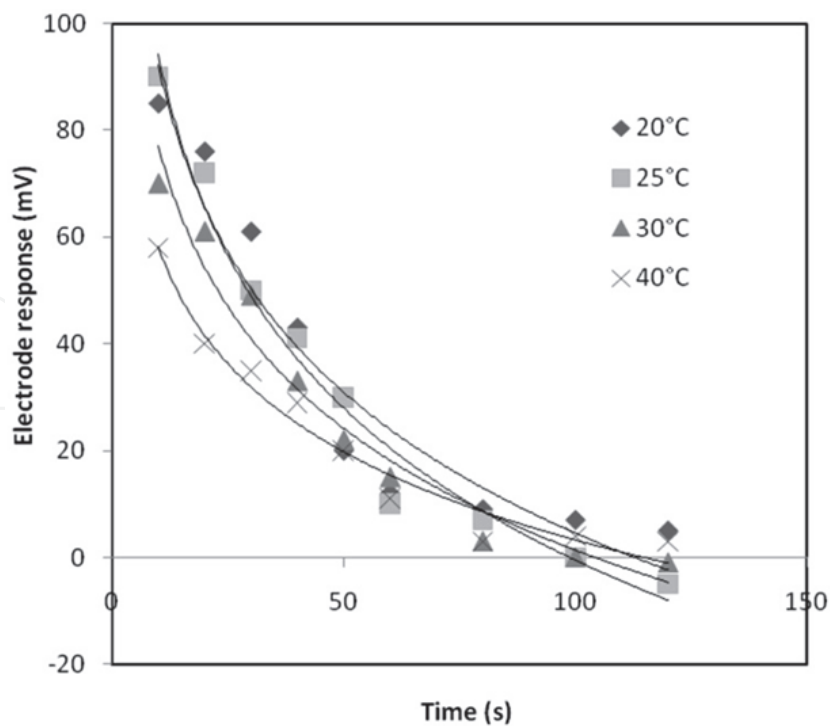

Fig. 9. Effect of temperature on the urea hydrolysis. Variation along the time of the ammonium ion-selective electrode (mV) response to a 2% (w/v) urea solution.

Furthermore, the effect of temperature of the buffer solution on the response of urea biosensor was studied in the range of 20–40°C. Figure 9 shows the ammonium ion-selective electrode against the buffer solution temperature. The 25°C was chosen the best temperature and utilized in further experimental investigations.

Through the tests using the fresh biocomponent, the best pH solution and test temperature were chosen. Furthermore, two jack beans amount (0.2 and 0.3 g) were chosen to be immobilized as Section 2.5 and used in the further tests. Although the 0.3 g jack bean weight has presented a better result as show in Figure 7, it was noted that 0.3 g did not have to be immobilized satisfactory results, and then the 0.2 g jack bean mass was also tested.

The powder of jack beans (urease source) was immobilized in different matrices (different screen materials), but the best results of mass retention were achieved with the screen nlyon (80% mass retention) (data not shown). So, this material was chosen to be used in the immobilization method in this work. Table 2 shows the values of ammonia concentration (ppm) generated by hydrolysis urea in 2 minutes of enzymatic reaction in experiments with 0.2 and 0.3 g of immobilized jack beans with urea solutions of 0.05% to 10.00% (w/v).

Ammoia concentration (ppm)		
Urea concentration % (w/v)	Jack beans weight (g)	
	0.2	0.3
0.05	62.13	42.29
0.10	258.61	205.31
0.50	30.24	243.77
1.00	525.73	271.94
2.00	675.31	371.47
4.00	981.44	533.27
6.00	1136.77	537.30
8.00	1200.35	583.23
10.00	1192.95	582.47

Table 2. Results of experiments to choose the best immobilized jack beans amount (0.2 or 0.3 g) analyzing the ammonia generation according Secion 2.4.

The table data are shown in the graph below (Figure 10). Figure 10 shows that the mass of 0.2 g had a higher urea hydrolysis into ammonia, whereas the immobilization of 0.3 g of jack bean on the nylon screen formed a thick film, hindering the mass transfer phenomena. So 0.2 g of jack bean was chosen as amount to be immobilized and utilized in further experimental investigations.

After the optimal pH of buffer solution, reaction temperature and immobilized amount of jack beans were chosen, the reproducibility of the ion-selective electrode response when inserted into 2% (w/v) urea solution was investigated under Section 2.11. Figure 11 shows a response variation, an average, of 11% over the eight times.
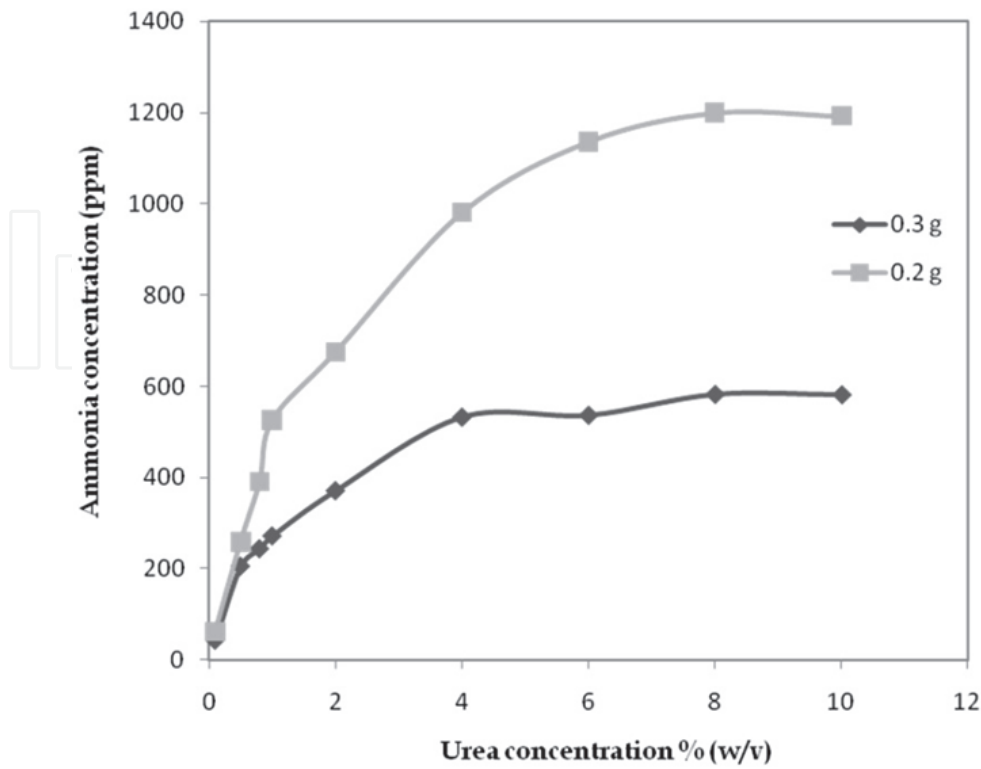
Fig. 10. Enzymatic reaction tests by jack bean immobilized mass variation (0.2 and 0.3 g), along the substrate (urea) concentration variation (0.05 to 10% (w/v)), pH 6.0, 25°C.
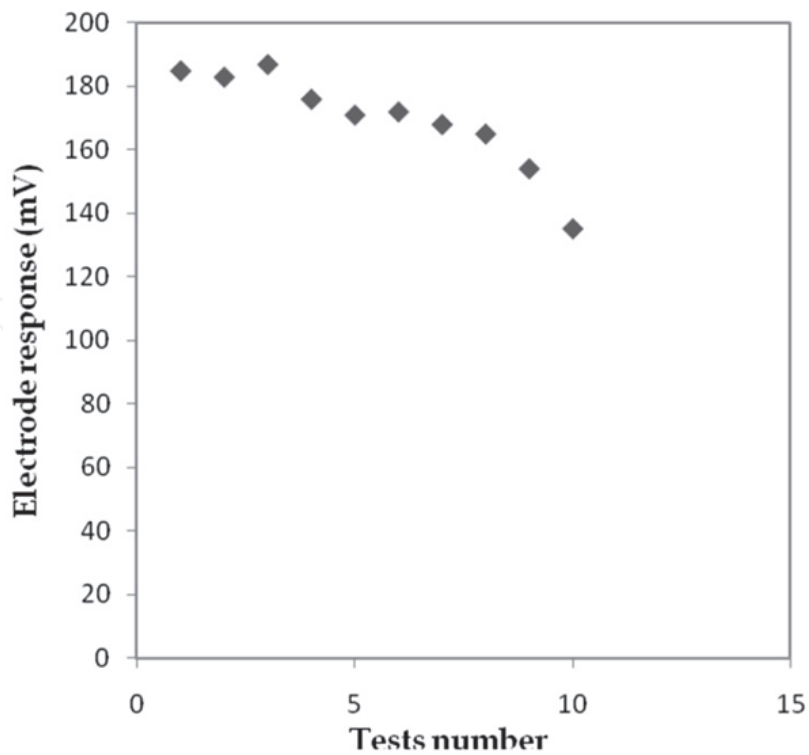

Fig. 11. Assay for reproducibility investigation according to Section 2.11.

3.2 Kinetic parameters determination

The kinetic parameters (K_m and V_{max}) were determined through the conventional Lineweaver–Burk plot, by varying the urea concentration between 0.05 to 10.00% (w/v) (Figure 12). The Km is an approximate measure of the affinity of the substrate for the enzyme. So the Km values for the free and immobilized ureases were also calculated. According to the results, the Km values were 19.10 mM and 411.39 mM for the free and immobilized urease, respectively.

This is a common result, because normally an increase of Km for an immobilized enzyme is expected. The difference of Km values suggests that substrate is easier to enter the active site of free urease because the enzyme immobilization may influence the diffusion of the substrate or product during reaction process. The K_m values found are within the range described in the literature (Verma & Singh, 2002) that lies between 2.08Mm for *Bacillus sp.* up to 100mM for *Bacillus pasteurii*. Therefore, it is noted that the study material (*Canavalia ensiformis* urease) has a good affinity with the substrate (urea) under the conditions tested.

Moreover, V_{max} values for the free and immobilized ureases were also calculated. According to the results, the V_{max} values were 3.76 mM/min and 31.26 mM for the free and immobilized urease, respectively. The value increase can be explained by enzymatic structure changes bye immobilization process that may have made the active sites more exposed to the substrate (urea).
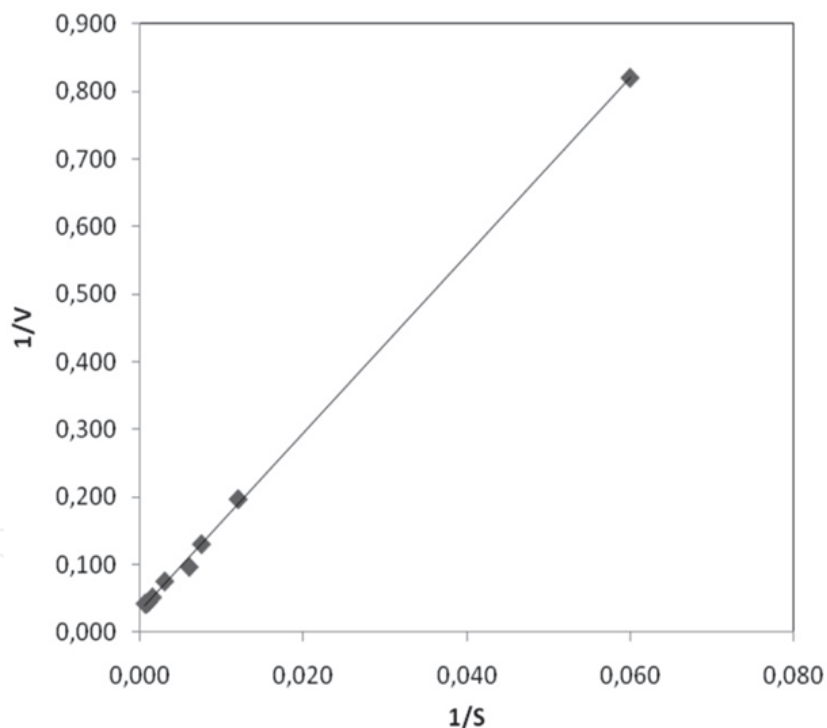

Fig. 12. Plot of 1/V against 1/[S] for immobilized urease (0.2 g of jack bean, pH 6.0, at 25°C).

3.3 Calibration curve of biosensor system and Reusability

The preliminary tests with the biosensor system were aimed in order to find the response time and linearity range of linearity of the instrument. For this, the range of 0.5 to 50.0 ppm of urea was chosen. Figure 13 shows that in all experiments, linearity range was observed in 1.0 to 20.0 ppm of the substrate. The best response time for the biosensor system was chosen 315 seconds. Moreover, water washing process between analysis samples did not cause

mass loss of immobilized biocomponent according to protein assay (Section 2.10). Calibration curve of protein assay is show in Figure 14.
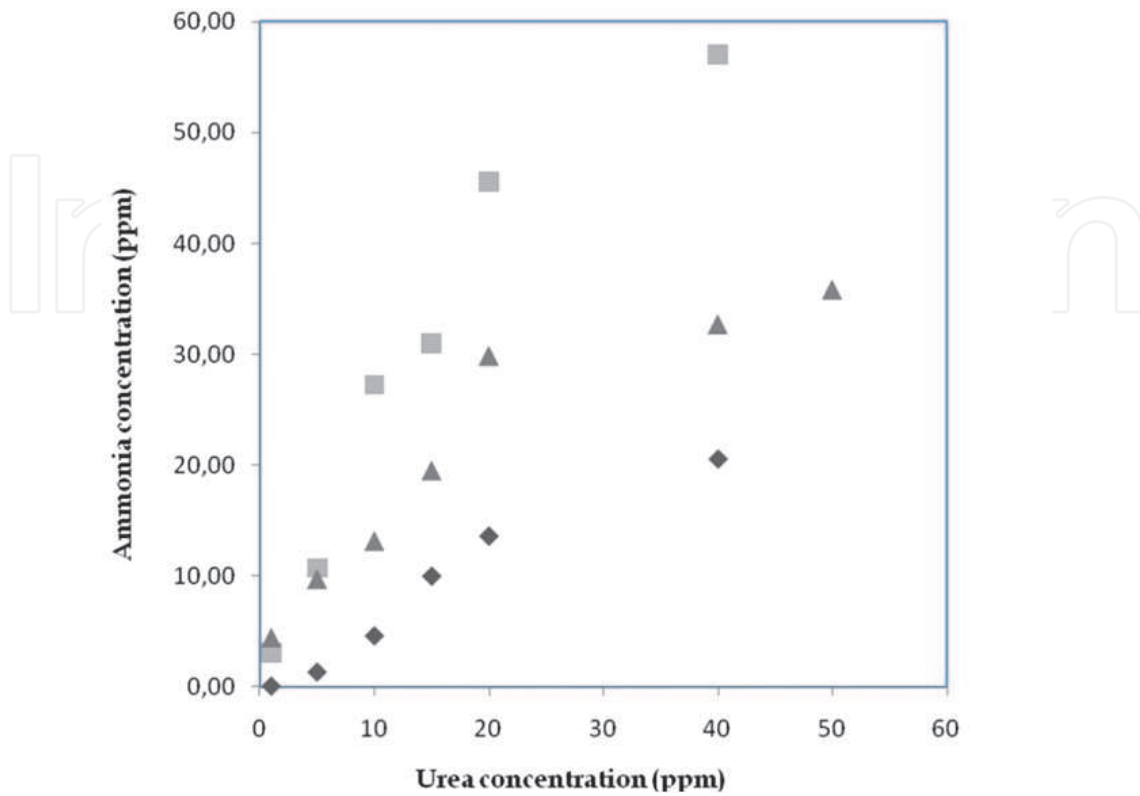

Fig. 13. Ammonia concentration variation over the urea range studied (0.5 to 50 ppm) using a biosensor system as a Section 2.8.1 for 317 seconds.
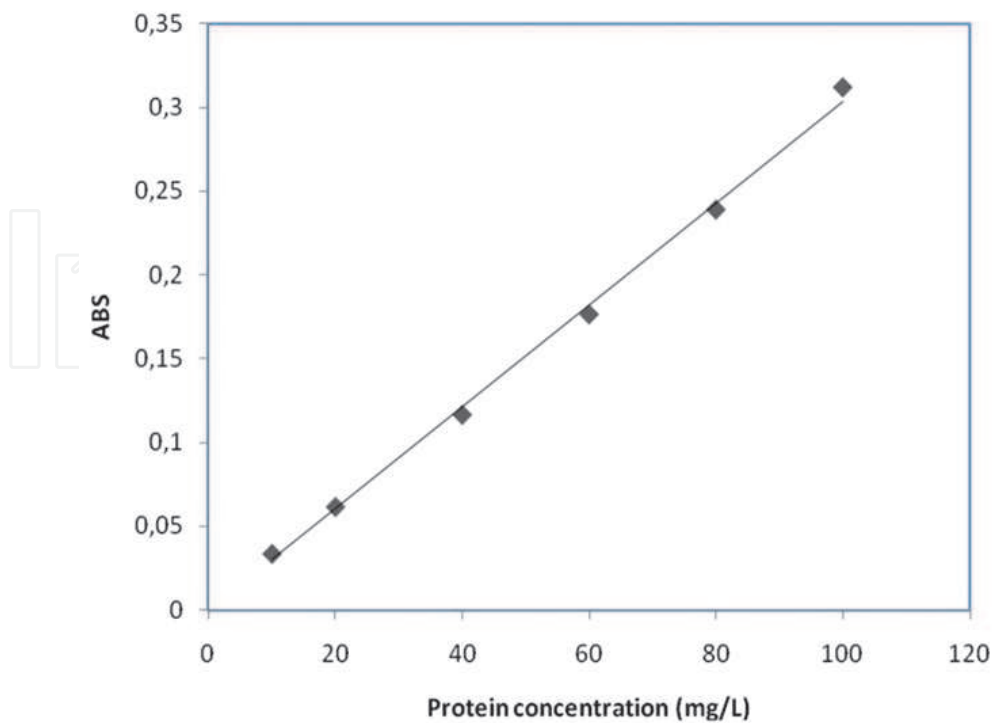

Fig. 14. Calibration curve of protein assay according Section 2.10.

Thereafter, to study the immobilized biocomponent reuse in biosensor system, assays were designed using the same immobilized urease along the days as Section 2.9.1. Figure 15 shows the results over 29 days using immobilized biocomponent in biosensor system. However, according to Table 3, it was found that up to 72 days of biocomponent use, the biosensor system has linearity range 1.0 to 20.0 ppm of urea, although the difference between the slopes of straight line, due to the urease activity of the immobilized jack bean powder.
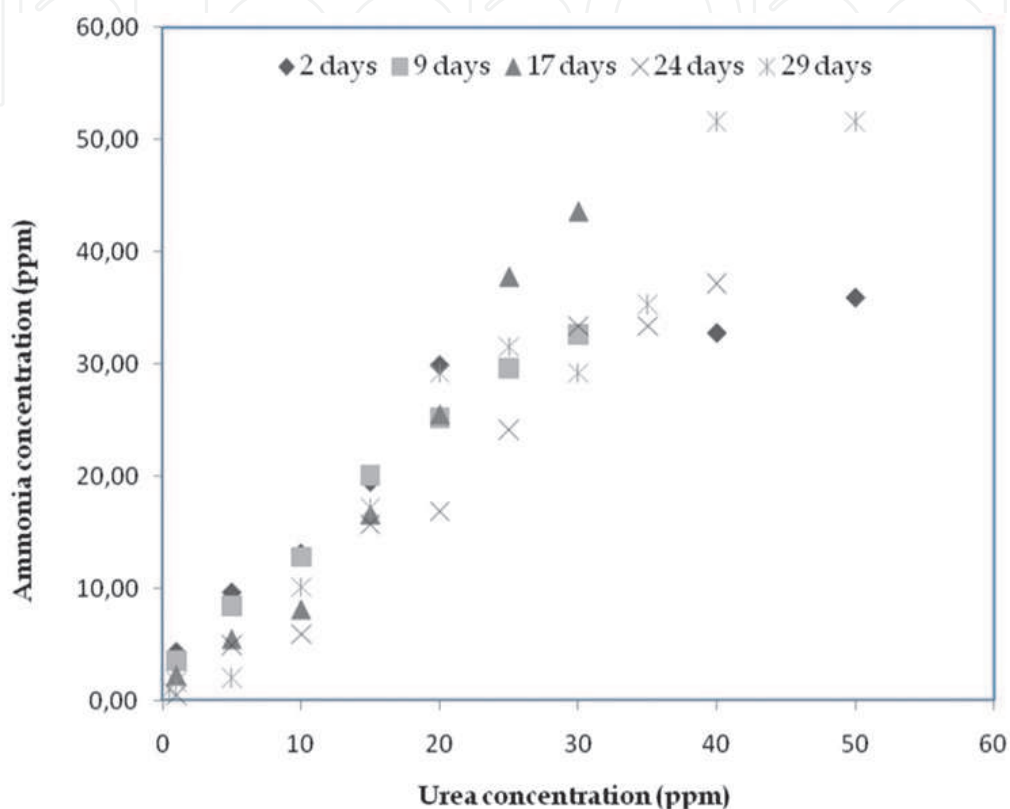
Fig. 15. Ammonia concentration variation over the urea range studied (0.5 to 50 ppm) using a biosensor system as a Section 2.8.1 for 29 days.

3.4 Storage stability

Figure 16 shows the storage stability of the three immobilized *Canavalia ensiformis* urease (as shown in Figure 4) stored at refrigerator (4°C) throughout the 1 month (30 days). The operational stability of a biosensor response may vary considerably depending upon the sensor geometry, method of preparation, biological recognition reactions etc. From Figure 16, it can be seen that the performance of the urea sensor stored under this conditions is not good. A pronounced decrease in the initial urease activity was observed over a nine days of stored.

4. Conclusion

In this study, for urease biosensor development, the urease was covalent immobilized on nylon screen by glutaraldehyde and the ammonia produced as a result of enzymatic reaction was monitored by potentiometry. The enzyme employed was from a rather non-

Storage time (days)	Linearity range of urea concentration (ppm)	Linear equation	R^2
02		y = 1.698x + 2.3518	0.9653
09		y = 1.1469x + 2.2988	0.9952
17		y = 1.2064x – 0,7344	0.9480
24		y = 0.9041x – 0,4318	0.9262
29	1-20	y = 1.4746 - 2,9956	0.9442
52		y = 2.0587x + 1,1672	0.9928
69		y = 0.963x - 2,2472	0.9699
72		y = 0.5912e0,13x	0.9908

Table 3. Characteristics of some calibration curves using system biosensor along the 72 days.
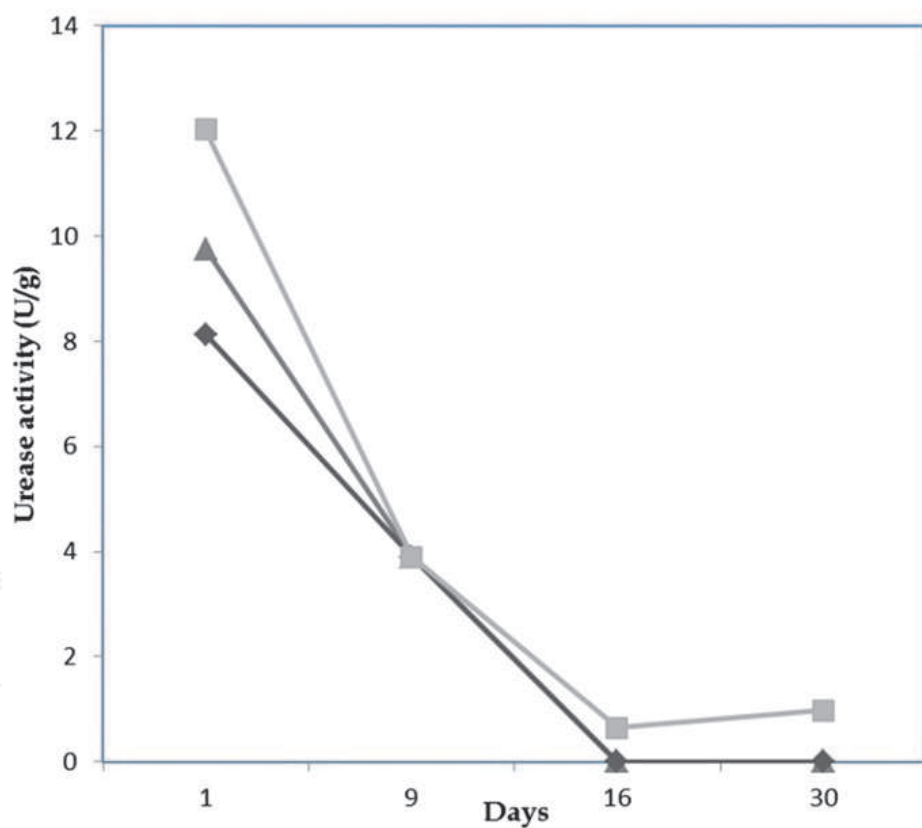
Fig. 16. Storage stability of the three immobilized *Canavalia ensiformis* urease stored at refrigerator (4°C) throughout the 1 month (30 days).

expensive and readily available source (jack beans). Some parameters had been analyzed aiming at to optimize the functioning of the biosensor. Through the results, in average, of storage the biological component activity declined more than 90% and the urea biosensor working range was 1-20ppm urea with a response time of 315s.

The developed instrument has become an inexpensive and practical tool for urea analysis in standard sample. Next step is biosensor application in environmental samples and industrial effluents, since it is necessary to monitor the nitrogen compounds concentration in waterbody to avoid problems such as eutrophication.

5. Acknowledgment

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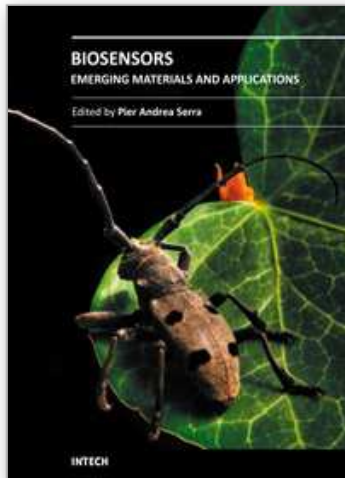
Biosensors - Emerging Materials and Applications

Edited by Prof. Pier Andrea Serra

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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