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# An amperometric oxalate biosensor based on sorghum oxalate oxidase bound carboxylated multiwalled carbon nanotubes–polyaniline composite film

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ABSTRACT

A highly sensitive, specific and rapid electrochemical oxalate biosensor was constructed by covalently immobilizing sorghum leaf oxalate oxidase on carboxylated multiwalled carbon nanotubes and conducting polymer, polyaniline nanocomposite film electrodeposited over the surface of platinum (Pt) wire using N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) chemistry. The modified electrode was characterized by scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectrophotometry. The optimized oxalate biosensor showed linear response range of 8.4–272 μM with correlation coefficient of 0.93 and rapid response within 5 s at a potential of 0.4 V vs Ag/AgCl. The sensitivity was approximately 0.0113 μA/μM with a detection limit of 3.0 μM. Proposed oxalate biosensor was successfully applied to human urine sample.



## 1. Introduction

Determination of oxalate in urine and blood is of great interest, as it is required in the diagnosis and medical management of idiopathic urolithiasis and various intestinal diseases (Archer et al., 1957). Various analytical methods reported for determination of oxalate are cumbersome, time consuming, less sensitive and require pretreatment of sample (Sharma et al., 1993). Therefore the demand for a simple, sensitive, accurate and rapid method has arrived. Over the past few years, various electrochemical methods for determination of oxalate based on the oxalate oxidase (OxOx) have been reported (Rahni and Guilbault, 1986; Assolant et al., 1987; Dinckaya and Telefoncu, 1993; Sezginturk and Dinckaya, 2003; Sakaa and Vallon, 1994; Shimohigoshi and Karobe, 1996; Reddy and Vadgama, 1997; Milardovic et al., 2000a,b, 2008; Perez et al., 2001; Hong et al., 2003; Fiorito and Torresi, 2004; Fiorito et al., 2005; Capra et al., 2007; Benavidez et al., 2009; Mishra et al., 2009; Pundir and Phaugat, 2009; Chaudhary and Pundir, 2010). However these OxOx based biosensors have some common drawbacks such as unstability due to leaching of enzyme from membrane/support, low conductivity and interference in electron communication. Therefore, there is a demand for the development of a biosensor that is stable, reproducible and sensitive for oxalate determination in real samples.

Carbon nanotubes (CNTs) has received a great deal of attention as an electrode material, because these have good electrocatalytic properties (Iijima, 1991; Cespedes and Alegret, 2000; Gooding, 2005) display metallic, semiconducting and superconducting electron transport and possess a hollow core suitable for storing guest molecules. These unique properties of CNT make it extremely attractive for chemical sensors, particularly for electrochemical biosensors (Tasis et al., 2006; Baughman et al., 2002). Such potential applications would benefit greatly from the ability of CNT to promote the electron transfer reactions of important biomolecules, including cytochrome c, catecholamine neurotransmitters, ascorbic acid (Wang et al., 2002a,b; Wang et al., 2002) and NADH (Musameh et al., 2002). Recently, the formation of polymer/CNT composites has been explored for possible improvement in the electrical and mechanical properties of polymers (Wei et al., 2002). CNT-based nanocomposites usually exhibit a higher conductivity than materials loaded with other fillers because of the high intrinsic conductivity and the high aspect ratio of CNT (Bocharova et al., 2006). Thus, conductive polymer/CNT composite film can be a useful platform for immobilizing biomolecules to enhance sensor performances. Among various conducting polymers, polyaniline (PANI) is unique due to its relatively facile synthesis, conductivity and environmental stability (Persaud and Pelosi, 1992; MacDiarmid et al., 1985; Skotheim et al., 1997). PANI/CNT composites have been prepared by electropolymerization of aniline (Gao et al., 2000;

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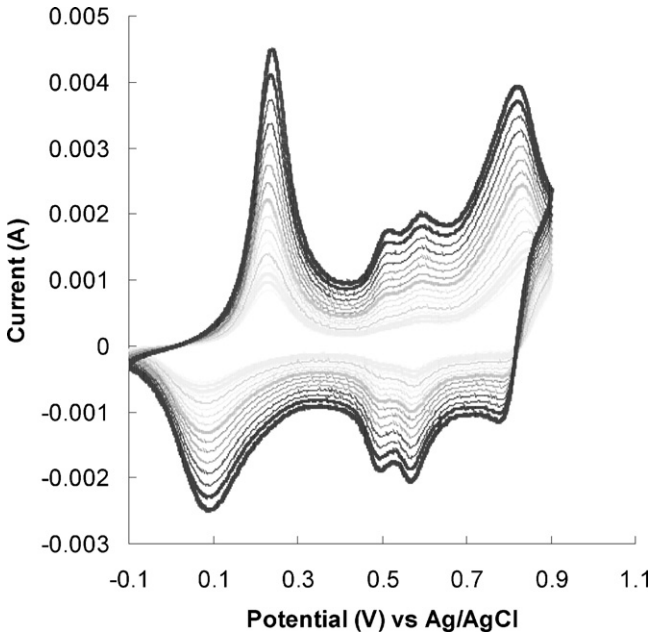



**Fig. 1.** Cyclic voltammogram for electrochemical deposition of c-MWCNT/PANI composite film on Pt wire. Supporting electrolyte: 1 N HCl solution; scan rate: 50 mV/s.

Huang et al., 2003; Du et al., 2009) or by in situ chemical polymerization (Cochet et al., 2001; Wei et al., 2003).

The present report describes construction and application of a carboxylated multiwalled carbon nanotube/polyaniline (c-MWCNT/PANI) nanocomposite based oxalate biosensor which provides covalent linkage between enzyme and electropolymerized c-MWCNT/PANI composite film on Pt wire using standard water soluble coupling agents N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) with an overall aim of improving the oxalate biosensor performance.

## 2. Materials and methods

### 2.1. Materials

Oxalic acid, Sephadex G-100, DEAE-Sephacel were from Sigma–Aldrich, USA. Carboxylated multiwalled carbon nanotubes (c-MWCNTs) (functionalized MWCNTs) were from Intelligent Materials Pvt. Ltd., Panchkula (Haryana), India. N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), Folin–Ciocalteu's phenol reagent, aniline (purified through vacuum distillation before use) were from SISCO Research Lab., Mumbai (India). The seeds of grain sorghum (Sorghum vulgar var. Amarnath) were gift from M/S Nath Seeds Private Ltd., Aurangabad, Maharashtra, India.

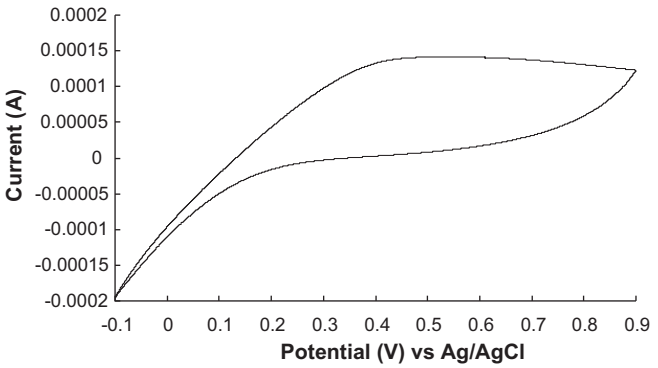



**Fig. 2.** Cyclic voltammogram for 10 mM oxalate solution in 0.05 M sodium succinate buffer (pH 5.0) with scan rate 50 mV/s.

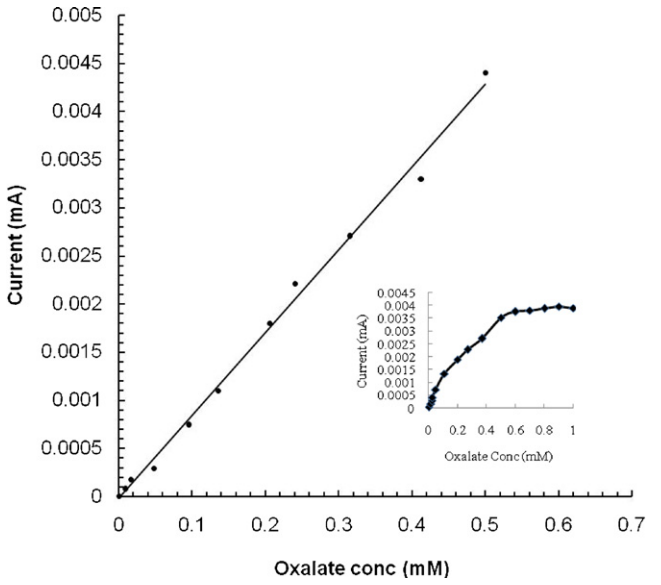



**Fig. 3.** Standard curve of oxalate by oxalate biosensor based on sorghum oxalate oxidase bound to c-MWCNT/PANI composite film electrodeposited on Pt wire.

### 2.2. Extraction, purification and assay of OxOx from sorghum leaves

Extraction and purification of OxOx from ten days old seedling plants of grain sorghum were done as described (Satyapal and Pundir, 1993). The activity of enzyme was measured by following the method of Satyapal and Pundir, while protein content was determined by Lowry method.

### 2.3. Construction of OxOx/c-MWCNT/PANI/Pt electrode

#### 2.3.1. Electrodeposition of c-MWCNT/PANI film on Pt wire

One milligram of c-MWCNT was suspended into 1 ml mixture of concentrated $H_2SO_4$ and $HNO_3$ in 3:1 ratio and ultrasonicated for 2 h to obtain a homogeneous mixture and washed. A solution for electrodeposition was prepared by adding aniline (50 μl) to 1 N HCl (10 ml) in a glass cell. Aniline was electrodeposited onto Pt electrode through electropolymerization using a Potentiostat–Galvanostat (Autolab, Eco Chemie, The Netherlands. Model: AUT83785). Prior to electrodeposition, the Pt wire (1.85 cm × 1 mm) was ultrasonicated in 5.0 M $HNO_3$ and acetone for 15 min and then rinsed with distilled water. The three electrode-system was immersed into the solution, the potential scan was cycled for 50 times between −0.1 and 0.9 V at a scan rate of 50 mV/s (Du et al., 2009). During the electrochemical polymerization, the surface of Pt wire became black gradually, indicating the deposition of PANI film on Pt wire (Fig. 1). The c-MWCNT/PANI film coated Pt wire was washed with deionized water and subsequently kept in a desiccator for 24 h at room temperature.

#### 2.3.2. Preparation of enzyme electrode

To prepare the enzyme electrode, purified sorghum leaf OxOx was immobilized covalently onto the c-MWCNT/PANI film by using EDC–NHS chemistry (Rahman et al., 2009a) as follows: first c-MWCNT/PANI/Pt electrode was immersed into a 0.1 M sodium phosphate buffer (PB) pH 7.0 containing 10 mM EDC and 10 mM

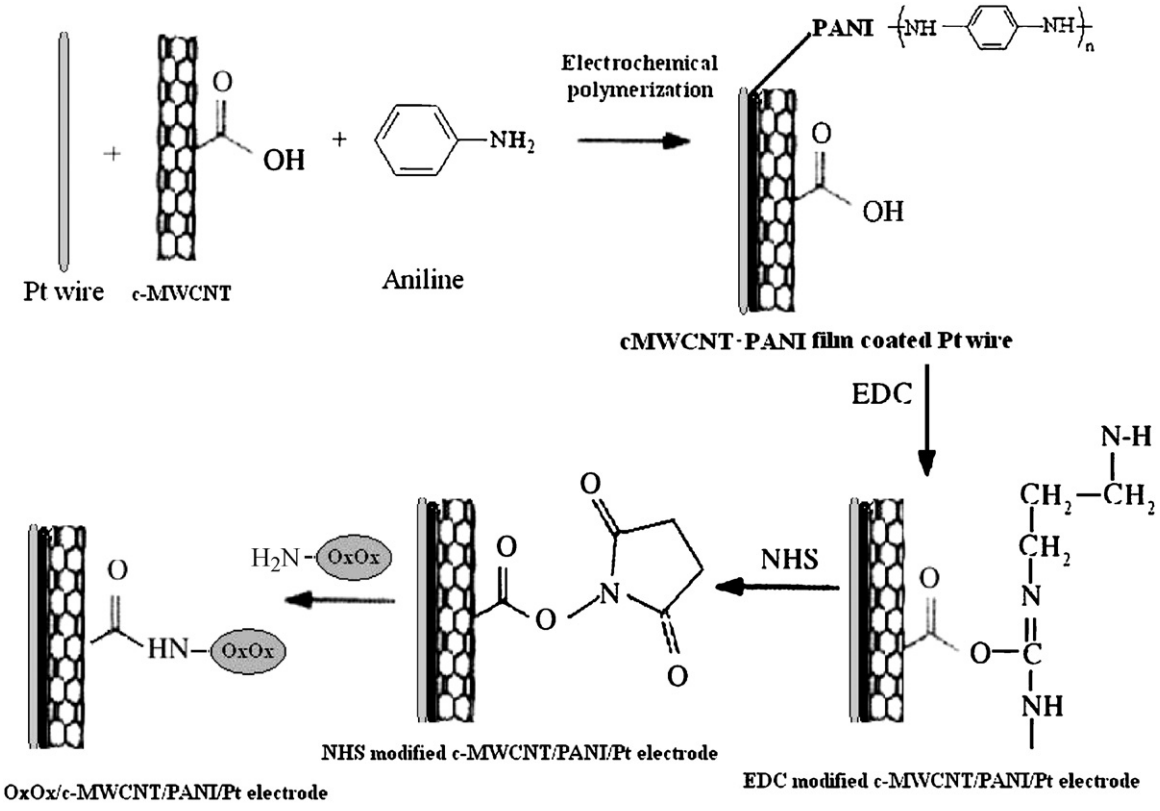



**Fig. 4.** Schematic representation of chemical reactions involved in fabrication of OxOx/c MWCNT/PANI/Pt electrode.

NHS for 6 h to activate the unbound and free –COOH groups of c-MWCNT/PANI composite film and then EDC–NHS treated electrode was washed three times with a 0.1 M PB, pH 6.8 to remove excess of EDC and NHS and finally incubated in 0.1 M PB, pH 7.0 containing purified OxOx (20 μg) at 4 °C for 3 h. The electrode was taken off the solution and washed in 0.1 M PB pH 6.8. The fabricated electrode was characterized by using SEM and Fourier transform infrared (FTIR) spectroscopy technique. Protein concentrations in remaining solution and wash out solution were determined. Enzyme electrode was stored at 4 °C when not in use.

### *2.4. Cyclic voltammetric measurement and testing of oxalate biosensor*

Cyclic voltammogram (CV) of OxOx/c-MWCNT/PANI electrode was recorded in Potentiostat–Galvanostat from −0.1 to 0.9 V vs Ag/AgCl as reference and Pt wire as counter electrode in a 0.05 M sodium succinate (pH 5.0) containing 10 mM oxalate. The maximum response was observed at 0.4 V (Fig. 2) and hence subsequent studies were carried out at this voltage. To test the functioning of the biosensor, three electrodes were connected to three terminal electrometer (Make: Keithley, model 6517A) and then immersed

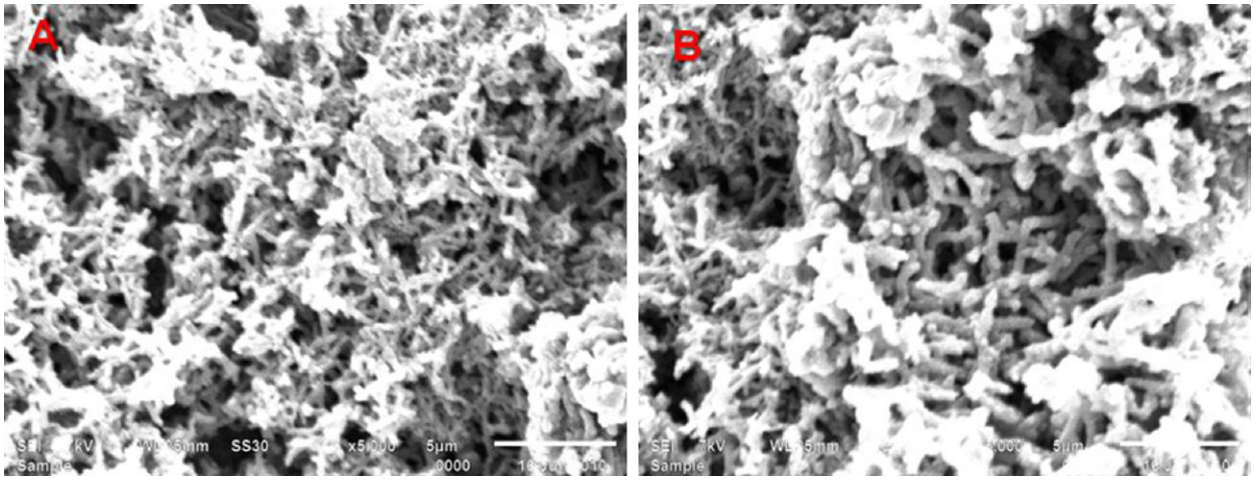



**Fig. 5.** SEM images of MWCNT/PANI (A) and OxOx/MWCNT/PANI (B) on Pt electrode.

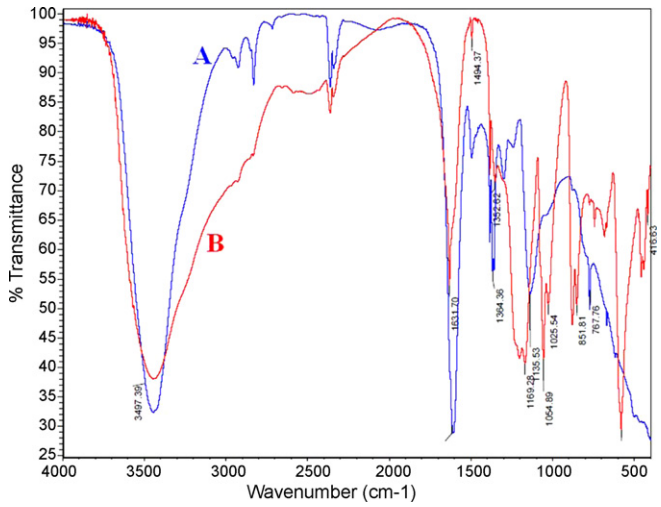



**Fig. 6.** FTIR spectra of MWCNT/PANI (A) and OxOx/MWCNT/PANI (B) on Pt electrode.

into 15 ml 0.05 M sodium succinate buffer pH 5.0 in a 50 ml beaker. The reaction was started by adding oxalate solution to reach a final concentration of 10 mM and the current (mA) generated was recorded at 0.4 V.

### 2.5. Optimization of oxalate biosensor

The experimental conditions affecting the biosensor response were studied in terms of effect of pH, incubation temperature, time and substrate (oxalate) concentrations. To determine optimum pH, the pH varied from 3.0 to 7.5 at interval of 0.5. Similarly optimum temperature was studied by incubating the reaction mixture at different temperatures (20–50 °C). The effect of oxalate concentration on biosensor response was determined by varying the concentration of oxalate from 3 μM to 1000 μM.

### 2.6. Urinary oxalate determination by oxalate biosensor

First morning urine samples from apparently healthy individual and stone formers of different age groups and sex from local area

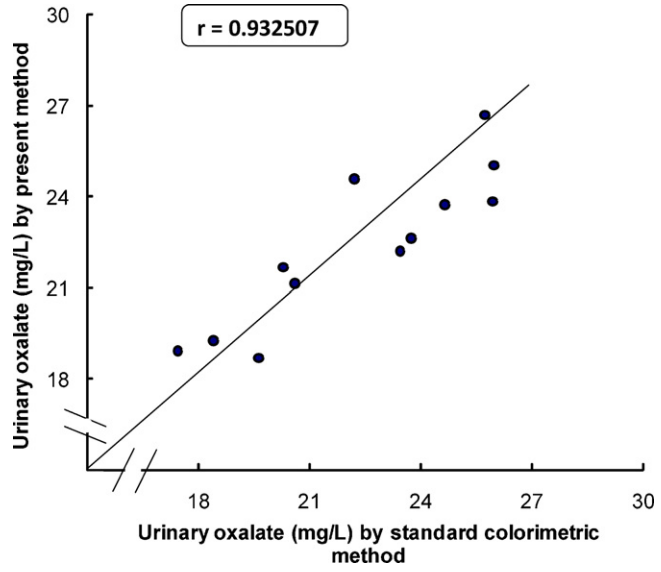



**Fig. 7.** Correlation between urinary oxalate values measured by standard enzymic colorimetric method ($x$) and present method ($y$) employing sorghum oxalate oxidase bound to c-MWCNT/PANI composite film electrodeposited on Pt wire.

were collected in tubes and stored at 4 °C until use. To determine urinary oxalate, the same procedure was used, as described above for testing of biosensor under its optimal working conditions except that oxalate was replaced by urine. The concentration of oxalate in urine was extrapolated from standard curve of oxalate between oxalate concentration and current (mA) prepared under optimal assay conditions (Fig. 3).

### 2.7. Evaluation

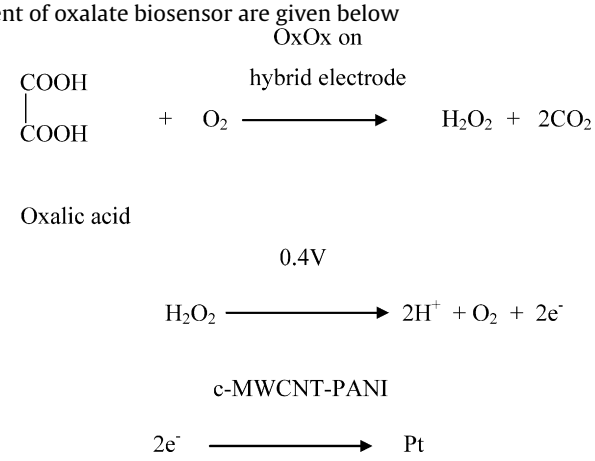
The following criteria was studied to evaluate the performance of this biosensor e.g. linearity, analytical recovery, detection limit, precision and correlation with standard method.

## 3. Results and discussion

### 3.1. Construction of OxOx/c-MWCNT/PANI/Pt electrode

A method is described for construction of an electrochemical oxalate biosensor using sorghum leaf OxOx immobilized covalently onto c-MWCNT/PANI film electrodeposited on the surface of Pt wire, as working electrode along with Ag/AgCl as reference and Pt wire as auxillary electrode. The chemical reaction involved in fabrication of the oxalate biosensor based on covalent immobilization of sorghum leaf OxOx onto the electrodeposited film of c-MWCNT/PANI on the surface of Pt wire is summarized in Fig. 4. In the polymerization process aniline exchanges electrons on the surface of c-MWCNT and binds with it by formation of charge transfer complex and then conducting c-MWCNT/PANI layer is deposited on the surface of Pt wire by the potential cycling method. This c-MWCNT/PANI composite film is expected to provide large surface area and catalytic activity to enhance the flow of current to Pt electrode. OxOx is immobilized on the c-MWCNT/PANI surfaces through the amide bond formation between the free –COOH groups of c-MWCNT/PANI film and the $-NH_2$ groups of the enzyme surface using the EDC–NHS chemistry. EDC and NHS activate the free –COOH groups of c-MWCNT/PANI composite layer.

The electrochemical reactions involved in response measurement of oxalate biosensor are given below




### 3.2. Surface characterization using SEM

The morphology of the modified electrode was characterized by SEM. Fig. 5 shows SEM images of Pt electrode coated with MWCNT/PANI and OxOx/MWCNT/PANI. The MWCNTs/PANI composite film shows net structure. Film is more uniform and porous and hence effective surface area is larger. The SEM images for OxOx/MWCNT/PANI show a different morphology from that obtained for the MWCNT/PANI layer, indicating the successful immobilization of OxOx to the surface of MWCNT/PANI layer.

**Table 1**
Analytical recovery of added oxalate in the urine samples, as measured by oxalate biosensor based on sorghum oxalate oxidase bound to c-MWCNT/PANI composite film electrodeposited on Pt wire.

| Oxalate added (mg/l) | Oxalate found (mg/l) | % recovery |
|---|---|---|
| – | 22.2 | – |
| 5 | 26.9 | 94.2 ± 2.3 |
| 10 | 31.6 | 95.1 ± 3.4 |

### 3.3. FTIR spectra

Fig. 6 shows FTIR spectra obtained for MWCNT/PANI/Pt electrode (curve A) and OxOx/MWCNT/PANI/Pt bioelectrode (curve B). FTIR spectra of electrodeposited MWCNT/PANI composite showed bands at 1460 and 1601 $cm^{-1}$ (curve A) which are caused by benzoid and quinoid vibrations. The presence of peaks at 1135.53 and 3448 $cm^{-1}$ attributed to β-$N^{+}$=Q and –N–H stretching vibrations of PANI in composite. In FTIR spectrum of OxOx/MWCNT/PANI/Pt bioelectrode (curve B) OxOx binding is indicated by appearance of additional bands at 1631.70 and 1494.37 $cm^{-1}$ assigned to the carbonyl stretch (amide I band) and –N–H (amide II band) respectively (Matharu et al., 2007).

### 3.4. Optimization of experimental conditions

The experimental conditions affecting the biosensor response were studied in terms of effect of pH, incubation temperature, time and substrate (oxalate) concentration. The optimum current was obtained within 5 s at pH 5 and 35 °C. There was a hyperbolic relationship between biosensor response and oxalate concentration up to a final concentration of 600 μM, after which it was constant. The Lineweaver–Burk plot between 1/*I* (mA) and 1/[oxalate] yielded a $K_m$ of 0.33 mM which is lower than that for free enzyme (7.8 mM) (Satyapal and Pundir, 1993) indicating increased affinity of enzyme towards substrate (oxalate) after immobilization, which might be due to enhanced diffusion of oxalate through c-MWCNT/PANI composite film (Rahman et al., 2009b).

### 3.5. Specificity

The relative response of biosensor was tested using following organic acid at a final concentration of 0.6 mM in place of oxalic acid: malonic acid, succinic acid, glycolic acid, glyoxylic acid, maleic acid, α-ketoglutaric acid, aspartic acid, glutamic acid, oxaloacetic acid, ascorbic acid, malic acid and citric acid. The sensor showed no activity with any other organic acid except oxalic acid in agreement with earlier report (Satyapal and Pundir, 1993).

**Table 2**
Within and between assay coefficients of variation for determination of urinary oxalate in the urine samples, measured by oxalate biosensor based on sorghum oxalate oxidase bound to c-MWCNT/PANI composite film electrodeposited on Pt wire.

| *n* | Oxalate (mg/l) | CV (%) |
|---|---|---|
| Within assay (5) | | |
| 22.2 | 20.9 | 5.1 |
| 20.6 | | |
| 21.8 | | |
| 19.7 | | |
| 20.2 | | |
| Between assay (5) | | |
| 22.3 | 21.34 | 5.34 |
| 21.5 | | |
| 19.8 | | |
| 20.6 | | |
| 22.5 | | |

### 3.6. Sample analysis of urinary oxalate

The oxalate content in urine samples, was in the range 11.56–15.69 mg/l in apparently healthy persons with a mean of 14.11 mg/l in males and 12.56 mg/l in females, but ranged from 17.44 mg/l to 26.01 mg/l in urinary stone formers with a mean of 24.95 mg/l in males and 19.79 mg/l in females.

### 3.7. Evaluation of oxalate biosensor

Analytical recovery of exogenously added oxalate in urine (5 mg/l and 10 mg/l) in the method was 94.2% and 95.1% respectively showing the reliability of the method (Table 1). The detection limit of biosensor was 3.0 μM, which is better/lower than that of any other earlier electrochemical oxalate biosensor (Sakaa and Vallon, 1994; Shimohigoshi and Karobe, 1996; Reddy and Vadgama, 1997; Milardovic et al., 2000a,b, 2008; Perez et al., 2001; Hong et al., 2003; Fiorito and Torresi, 2004; Fiorito et al., 2005; Capra et al., 2007; Benavidez et al., 2009; Mishra et al., 2009; Chaudhary and Pundir, 2010) with the exception of DO metric oxalate biosensor based on PVC membrane (1 μM) prepared in our laboratory (Pundir and Phaugat, 2009) which had a drawback, interference by aerobic $O_2$. In order to check the repeatability and reproducibility of the method, the oxalate content in same urine sample was determined five times on a single day (within batch) and again after storage at −20 °C for one week (between batch). The results showed that determinations were almost consistent and within and between batch coefficients of variation (CVs) for urinary oxalate determination was <5.1% and <5.34%, showing good reproducibility and reliability of the method (Table 2). To know the accuracy of present method, values of oxalate in first morning urine sample obtained

**Table 3**
A comparison of various electrochemical oxalate biosensors with c-MWCNT/PANI composite film based oxalate biosensor.

| Properties | Mishra et al. (2009) | Chaudhary and Pundir (2010) | Present work |
|---|---|---|---|
| Source of oxalate oxidase | Sorghum leaves | Sorghum leaves | Sorghum leaves |
| Support for immobilization | Carbon paste electrode | CA membrane | c-MWCNT/PANI composite film |
| Methods for immobilization | Adsorption | Cross-linking | Covalent |
| Type of transducer | Amperometric | Amperometric | Amperometric |
| Working optimum pH | 4.5 | 5.5 | 5.0 |
| Optimum temperature (°C) | 35 °C | 35 °C | 35 °C |
| Response time | 45 s | 2 min | 5 s |
| Linearity | 0–222 μM | ND | 8.4–272 μM |
| Detection limit | 27.77 μM | 20 μM | 3.0 μM |
| Correlation with standard method | 0.91 | 0.98 | 0.93 |
| Storage life | 35 days | 30 days | >50% in 100 Days |
| Number of reuses | 150 times | 300 times | 120 times |
| $K_m$ | ND | $0.33 \times 10^{-3}$ M | $0.33 \times 10^{-3}$ M |
| Application: oxalate determination in | ND | Urine | Urine |
| Interference by | ND | No effect of Mn, Mg, Ca, Na, etc. | ND |

from apparently healthy and stone formers were determined by Sigma enzymic colorimetric method ($x$) and by the present method ($y$). The urinary oxalate values obtained by the present biosensor matched with enzymic colorimetric method with a good correlation ($r = 0.93$, significant at 1% level) (Fig. 7).

### 3.8. Long-term stability of electrode

To examine the long-term storage stabilities, the activity of c-MWCNT/PANI/OxOx/Pt electrode was tested with respect to storage time. After each experiment, the sensor was washed with buffer solution and stored at 4 °C. Studies revealed that the electrode retained more than 50% activity even after 100 days of regular 120 uses.

A comparison of different parameters of present oxalate biosensor with those of earlier oxalate biosensors is summarized (Table 3).

## 4. Conclusion

An improved amperometric oxalate biosensor was constructed by covalent immobilization of sorghum OxOx on conducting c-MWCNT/PANI composite film electrodeposited on Pt electrode. The biosensor was highly specific, more rapid, sensitive and stable than earlier biosensors.

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