

Cite this: *Anal. Methods*, 2011, **3**, 1981

www.rsc.org/methods

PAPER

An ultra sensitive method for rapid *in vitro* catalase assay with software based approach using LabVIEW virtual instrumentation†

Sandeep Kumar Jha‡* and Stanislaus F. D'Souza

Received 16th December 2010, Accepted 8th June 2011

DOI: 10.1039/c0ay00760a

A novel approach for estimation of catalase activity in biological samples was developed using real-time signal averaging with LabVIEW based virtual instrumentation. The assay was performed by on-line monitoring of the increase in dissolved oxygen concentration due to the catalytic reaction of the enzyme with its substrate H_2O_2 using a Clark type dissolved oxygen electrode. The electrode was interfaced with a computer using an op-amp based electronic circuit and LabVIEW based software for signal acquisition, data visualization and signal conditioning. Catalase activity in biological samples could be measured without pre-dilution and the method was not affected by interferences such as turbidity, viscosity and sample color. It was possible to increase the assay sensitivity from 0.5 U for the existing spectrophotometric method to 2.93 μU , with a sample size as little as 100 μl and response time as low as 9 s. Therefore, the proposed method shall find use in clinical assays, detection of pathogens and evaluation of catalase activity in cell cultures, neurological samples, milk and other food products.

1. Introduction

The enzyme catalase (E.C. 1.11.1.6) can be regarded as a biochemical marker in many applications, including detection and identification of catalase positive pathogenic organisms such as *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Campylobacter jejuni* and *Listeria*, or catalase negative haemolytic *Streptococcus pneumoniae* by enzymatic assay based biochemical and physiological characterization in clinical laboratories.¹ Apart from this aspect, catalase is regarded as a useful biomarker in the study of apoptosis;² oxidative stress and reactive oxygen species (ROS);³ the effect of heavy metals, elemental sulphur and mercury on catalase activity in the liver and the *in vivo* effects of UV and γ -rays. It is used to monitor the *in vivo* effect of drugs such as isoniazid and as an indicator in tissue biopsies⁴ of the liver, spleen, heart, brain *etc.* Catalase is also an indicator for diseases such as phenylketonuria,⁵ acatalasia⁶ and gastric ulcers.⁷

The enzyme catalase is known to produce one of the fastest enzymatic reactions in biological systems with a turnover number of more than 10^7 s^{-1} . Therefore, reaction of this enzyme with H_2O_2 results in vigorous evolution of oxygen bubbles. Traditionally, there are several direct and indirect methods for measuring catalase activity. For example, spectrophotometric

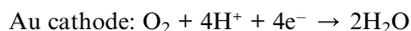
measurement of H_2O_2 at 240 nm;⁸ ferrous and thiocyanate ion based spectrophotometric determination of hydrogen peroxide suitable for plate reader;⁹ Clark oxygen electrode based catalase assay applicable to the kinetic study of the pea leaf enzyme;¹⁰ spectrophotometric assay for serum catalase activity based on a stable chromophoric complex of H_2O_2 with ammonium molybdate¹¹ or determination of a stable chromogenic complex of methanol with hydrogen peroxide, which forms formaldehyde that can be detected spectrophotometrically by 4-amino-3-hydrazine-5-mercapto-1,2,4-triazole (Purpald) chromogen.¹² More recently, a few attempts have been made to redefine the assay method for catalase to suit different samples and methods. Huidobro *et al.* used an assay system containing *o*-dianisidine and peroxidase for the determination of H_2O_2 spectrophotometrically at 400 nm.¹³ Still, most of the existing methods for the estimation of catalase face the drawbacks of a low accuracy of measurement because of the high turnover rate of catalase, and the human-induced error of having a time lag between the addition of H_2O_2 and measurement of the optical density. These methods are less sensitive because of instrumental limits. Secondly, detection of hydrogen peroxide at 240 nm cannot be performed in turbid tissue preparations. Also, evolution of oxygen bubbles due to enzymatic reaction causes interference.⁸ Thus, it is desirable to develop an interference free, rapid and sensitive assay protocol for catalase.

To overcome these problems, a new sensor based estimation method for catalase activity was developed and described in the present work. The reaction between the enzyme catalase and hydrogen peroxide yields molecular oxygen, which can be measured using a Clark type, dissolved oxygen probe with the following electrodic processes.

Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, 400085, India. E-mail: sandeepkja@gmail.com; Tel: +82-10-4966-4194

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c0ay00760a

‡ Present address: Department of Nanoscience & Engineering, Myongji University, Yongin, Gyeonggi, South Korea 449-728



For this purpose, a sensitive dissolved oxygen (DO) meter was designed to enhance the sensitivity of detection using a combination of low cost operational amplifier (op-amp) based electronic circuit and high precision analog to digital conversion and software based signal conditioning. Catalase activity was calculated from the slope of the linear portion of the sensor signal. The assay methodology, possible implications of this method in cell cultures, neurological samples, milk and other food products, clinical assays and pathogen detection are described in the present work.

2. Materials and methods

2.1. Materials

A dissolved oxygen probe was purchased from Century Instruments, Chandigarh. Software LabVIEW and a 16-bit ADC card, model NI 6013 were from National Instrument, USA. I/O connector for ADC card was purchased from Theta Control. Stabilized hydrogen peroxide was bought from Glaxo. Purified catalase from bovine liver was from Sigma Aldrich, USA. The electronic components were purchased from a local market.

2.2. Methods

The dissolved oxygen meter, flow through reaction vessel, data acquisition program and assay methodology were developed as described in the following sections.

2.2.1. Designing a flow through cell for the assay. A flow-through reaction vessel was designed using a plastic cylinder drilled and fitted with surgical tubes to regulate the flow of buffer from the external buffer tank using siphon action. A DO probe containing a silver anode and gold cathode (Fig. S1, ESI†) along with plastic casing and Teflon membrane, separating the electrode surface from external media was inserted from the top-hole of the vessel. The DO probe was filled with a freshly prepared solution of 1 M KNO_3 to maintain electrolyte conduction. The total volume of liquid inside the reaction vessel was maintained at 10 ml. A small magnetic bead was placed inside the vessel for continuous mixing of solution. Small amounts of samples (100 μl) could be added using an autopipette through a small hole made at the top of the reaction vessel.

2.2.2. Design of the electronic circuit. The dissolved oxygen probe was attached to a current to voltage (I - V) converter op-amp circuit (Fig. 1) to convert the output current from the electrodes into potential. Further, an op-amp inverter was used to amplify the signal tenfold and to change its polarity for ease in calculations. The power source of the electronic circuit was stabilized using integrated circuit (IC) based regulators. IC series 7805 and 7815 were used to maintain +5 and +15 V, whereas 7905 and 7915 were used for -5 and -15 V respectively. This minimized the ripple in the input potential ($+V_{\text{CC}}$ and $-V_{\text{EE}}$) to the op-amp. The instrument's operable range was ± 10 V.

An additional RC (resistor and capacitor) circuit was used to maintain a cathodic potential of +0.7 V across the electrodes in the DO probe.

2.2.3. Software development and interfacing of analog electronic circuit to a computer. A 16-bit ADC card from National Instruments was used to acquire the waveform from the analog circuit. The analog potential output from the second op-amp of the DO meter was captured by one out of eight available input channels in the ADC card with single ended non-referenced pin connection at a scan rate of 30 000 per second.

A program was developed using LabVIEW for interfacing the electronic circuit with the computer and for data visualization, signal conditioning and analysis (see Fig. S2 and S3, ESI† for block diagrams of the code and guided user interface, GUI, of the developed software). The data points acquired using this program were summed every second and averaged by dividing it by the scan rate. The computer's internal clock was used as a reference for time. These averaged data points were plotted in real-time as per straight-line equation and recorded in ASCII (text) file for further use. The calibration data thus obtained was fed into the program to convert the averaged output potential into DO concentration in parts per million (ppm). The program had an option to calculate the slope and intercept of the straight-line sensor response. A textbox was placed in the user interface to enter the values of the slope of response curve from the sample and control manually, so that their difference could be used to calculate catalase activity.

2.2.4. Calibration of instrument against commercially available DO meter. The DO meter was calibrated with respect to a commercially available instrument from Orion, Inc. USA. For this purpose, a DO probe from Century Instruments was filled with 2 ml of a freshly prepared solution of 1 M KNO_3 . The experiments were performed at room temperature (28 °C) and probes for both the instruments were maintained under similar conditions. Nitrogen or oxygen was purged in distilled water to record the dissolved oxygen level. For this purpose, 20 ml of distilled water was stirred in a 25 ml beaker at 300 rpm using a small magnetic bead. Oxygen was purged into it at 0.5 Kgf cm^{-2} pressure for 20 min. Nitrogen was purged in another set of experiments at 2.5 psi pressure for 30 min. To maintain zero oxygen concentration, 1 ml solution containing 500 mg of pyrogallol dissolved in 1 M NaOH was added to this stirring distilled water. The DO probe was immersed in the stirring gassed water and potential or DO concentration were recorded with respect to time.

2.2.5. Standardization of catalase assay using the developed sensor. The concentration of H_2O_2 in the stock reagent was determined by titration against 1 mM KMnO_4 to the greyish-green end-point. This stock solution was diluted to a working concentration of 34.095 mM with 50 mM phosphate buffer. Dilutions were prepared afresh to avoid contamination from dissolved atmospheric O_2 . Catalase activity was measured for the pure enzyme stock solution using a spectrophotometric method.⁸ Definitions of enzyme activity and specific enzyme activity of catalase as accepted were the amount of enzyme necessary to liberate 1 μmole of O_2 in 1 min at 30 °C¹⁴ and micromoles of H_2O_2 degraded per minute per mg of enzyme, respectively.

pulse mode. The cathodic potential was responsible for reduction of oxygen molecules that have crossed the Teflon membrane because of the difference in pO_2 . The electric current generated in this process was proportional to the amount of O_2 inside the KNO_3 -filled DO probe and so to the DO concentration in the flow through cell. The relation between them can be given by the Cottrell equation.¹⁸

The dissolved oxygen probe was attached to a current to voltage (I - V) converter circuit (Fig. 1). With this arrangement, the instrument was operational in the output range of ± 10 V. The developed circuit had an analog output and low signal to noise ratio, making it highly desirable for post-acquisition signal conditioning. This was achieved by interfacing the circuit with a computer and performing software based signal conditioning. Therefore, potential output from the second op-amp was channelled into the ADC card through the I/O connector bridge. The analog to digital conversion¹⁹ was necessary for the presentation and analysis of the signal from the analog electronic circuit with the LabVIEW program.²⁰

3.2. Software development and interfacing of DO meter with computer

The program required for acquisition of the analog signal was developed using LabVIEW software. The program had provisions for defining channel number, device number, scan rate, refresh rate, *etc.* and data could be plotted on-line in a graph placed next to the control panel of the GUI (Fig. S3, ESI†). The program was developed by joining individual precompiled modules from the LabVIEW library, which upon execution allowed signal acquisition, display and calculation of response slope. Further, to minimize the fluctuations in the output signal, the acquired data was averaged using the program and plotted per second on the GUI. The maximum theoretical sampling rate of the ADC card was 200 kHz. Averaging the signal at different sampling rates produced different patterns of the output waveform. A sampling rate of 30 kHz was found to be optimal based on the lowest fluctuation of signal from the baseline, still providing a large number of data points per second. Therefore, one data point represented an average value of 30 000 acquisitions per second and it was displayed as an on-line graph. This was perhaps the first instance of any such signal averaging technique used in biosensor measurement. By using this software, the sensor response curve was obtained for either evolution of O_2 upon catalase- H_2O_2 reaction or escape of O_2 from solution between successive experiments or washings.

3.3. Calibration of DO meter against commercially available instrument

The DO meter was interfaced with the computer using the acquisition program and was calibrated against the commercially available instrument from Orion Inc. The dissolution or evolution of O_2 from water at room temperature (28 °C) by either purging N_2 or O_2 produced a potential change that was proportional to the DO concentration. The potential values were recorded with time. The dissolved oxygen concentration in water varied over time as recorded with the commercial DO meter (Fig. S5, ESI†). A similar trend was observed for the developed

DO meter, except that it showed sensor response in terms of potential. A direct linear correlation existed between potential values from the developed instrument and the DO concentrations obtained using the commercial instrument ($V = 0.30789$ DO, ppm + 0.42531), as both the DO probes were maintained under similar conditions (Fig. 2). The coefficients (slope and intercept) of this correlation were fed into the data acquisition program to interpret output potential directly as DO concentration. The highest detection limit of the instrument was observed as 20 ppm of DO, beyond which the sensor response showed saturation, mainly due to the limitations of the op-amp circuit. The sensitivity of the instrument was calculated as 0.30789 V/ppm of DO at a signal to noise ratio in control (in plain buffer) of 3. At this level of sensitivity, the theoretical resolution of the instrument was calculated as 494 ppt (parts per trillion) of DO, while considering the resolution of the 32 bit ADC card as 152.5 μ V at an input potential range of 0–10 V. Another uniqueness in the present study was the ability to perform real-time calculations up to ninth decimal of ppm *i.e.* 10^{-3} ppq (parts per quadrillion), with the help of the data acquisition program which assisted in accurate signal averaging and analysis.

3.4. Assay and standard curve for catalase

The DO meter was calibrated for the assay of catalase and the standardization was followed using fixed substrate strength and varying catalase concentrations. The addition of the enzyme to the medium containing buffer and H_2O_2 showed a rise in the DO level (Fig. S6, ESI†). The time required for each assay depended on catalase concentration and typically varied between 9–60 s. The DO level reached the baseline after complete consumption of H_2O_2 and subsequent stirring of solution or upon washing the DO probe with a flow of buffer. The electrode could be regenerated within 60 s for successive assays through washing with buffer. These observations proved the feasibility of the sensor-based assay of catalase.

The catalase activity in the stock sample was first determined through an assay using the spectrophotometric method.⁸ The experiment was repeated 10 times, while monitoring optical density (OD) based enzyme kinetics. The linear portion of the

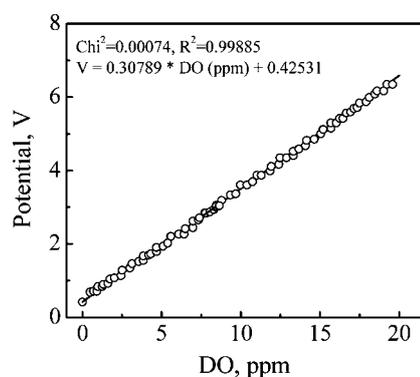


Fig. 2 Correlation between the DO concentration and response from the DO meter in terms of potential. Straight-line equation as obtained by curve fitting using Origin™ software: $V = 0.30789$ DO (ppm) + 0.42531 with $\chi^2 = 0.00074$ and $R^2 = 0.99885$.

kinetics curve was used to calculate the enzyme activity. A mean of all the values was calculated as 3535163.5 U ml⁻¹ catalase activity, while the assay was performed within the detection limit of the spectrophotometric method.

For obtaining a standard curve for catalase assay, H₂O₂ was first added in the reaction vessel and buffer was stirred until a baseline response was obtained. Subsequently, catalase was serially diluted in phosphate buffer and added to the vessel. A sigmoidal sensor response curve was obtained with a lag time, which varied with enzyme concentration used. The lag time was in the range of 2–9 s for the entire detection range. After the lag period, the sensor response increased in a linear fashion and saturated to the instrument's upper detection limit of 20 ppm of DO. The time required for saturation also depended on catalase activity.

The response curves were steeper for higher concentrations of catalase, indicating a direct correlation between this slope and the enzyme activity. The sensor response still had a degree of noise, therefore instead of directly correlating DO concentration to catalase activity, the slope of the linear portion of the response curve was chosen as a function to calculate enzyme activity. This novel approach not only improved the sensitivity and limit of detection (LOD)²¹ of the assay method to manifolds, but also improved the signal to noise ratio.

The response time for the assay was determined from patterns of multiple assays for 2.93 μU to 0.293 μU of catalase activity in 10 ml reaction volume, and 9 to 60 s was found to be a sufficient interval to determine the slope of the response curve. For catalase concentrations any higher than this value and up to 2930 U 10 ml⁻¹ reaction mixture, 3–10 s response time was found suitable for calculation of the slope. For catalase concentrations above this level, 2–6 s was used for the determination of the slope. The standard curve for the assay of catalase activity is shown in Fig. S7, ESI†. From this figure, it is evident that the enzyme activity was correlated with sensor output in a highly exponential manner. Such a dramatic non-linear response from the sensor can be attributed to the enzyme kinetics of catalase. This enzyme does not exhibit Michaelis–Menten kinetics with its substrate H₂O₂²² and does not become saturated with substrate. At high H₂O₂ concentrations, catalase exhibits lower reaction velocity than predicted by the Michaelis–Menten equation because of inactivation by H₂O₂.²² Under these circumstances, a sigmoidal-shaped sensor response curve is expected. However, the response curve observed for this sensor followed exponential behaviour (Fig. S7, ESI†), since catalase concentrations above 29308 units/10 ml reaction volume could not be assayed due to highly vigorous reactions. On the other hand, the effect of factors such as mass transport and diffusional constraints²³ or non-isothermal conditions,²⁴ which are generally associated with non-linear biosensor response, should be negligible in the present case as the reaction vessel for sensor measurement was maintained at room temperature and constant stirring throughout the study. Therefore, for the sake of simplicity and ease in calculations, a logarithmic axis was adopted for the sensor calibration curve, with the X-axis as log [ΔSlope, (δDO in ppm sec⁻¹)] and the Y-axis as the logarithm of catalase concentration in mU per 10 ml reaction volume. With such provision, the standard curves for each detection range could be maintained in the form of straight lines.

The simplified standard curve for assay of catalase activity is shown in Fig. 3, which had four detection ranges. Region A had lowest detection range (2.93 μU to 29.3 μU in a 10 ml reaction volume); followed by region B (29.3 μU to 0.293 U) and region C (0.293 U to 2930 U). Region D corresponded to the highest detection range of 2930 U to 29 300 units of catalase in 10 ml reaction volume. The straight-line equations of the curves in regions A–D were $Y = 0.6140269 X - 0.61598$; $Y = 5.93125227 X + 7.18023$; $Y = 2.60914275 X + 4.5922$ and $Y = 26.5995703 X - 12.29126$, respectively. The coefficients (slope and intercept) of these equations were fed into the acquisition program using a case loop. However, in this manner, it became essential to have a rough knowledge of catalase activity in the sample before its quantitation, in order to select the proper fitting range in the acquisition program. Thus, for an unknown sample, the assay should be performed at least twice: once to know the rough estimate of enzyme activity by observing the slope of the response curve and then selecting the appropriate detection range from the drop down menu placed on the GUI for the subsequent estimation. The case loop, therefore, acted as a switch to perform different calculations as per the conditions. For these reasons, two separate graphs were placed on the program, one to visualise the slope of the curve and the second to interpret the result in terms of enzyme activity. For simplicity, the drop-down selection menu had been set to choose slope range instead of enzyme activity. Once the acquisition program was fully functional with added coefficients for calculation of catalase activity in samples, the sensor-based assay was compared with the spectrophotometric method by assaying catalase in synthetic samples with known enzyme activity. The comparison between data obtained by both the methods is shown in Fig. 4. The straight line in the figure with unit-slope represents ideal agreement between the measurement methods being compared. As is evident from the figure, the data points with the error bars nearly follow an ideal agreement, with the coefficient of variation calculated as 5.9%. This value indicates accurate prediction of catalase test results, while a typical coefficient of variation is 3.8% for a commercially available catalase assay kit, that uses optical assay from tissue homogenates (Cayman Chemical, Michigan, USA, Item Number 707002). However, the required degree of accuracy for a catalase assay may vary between samples and by

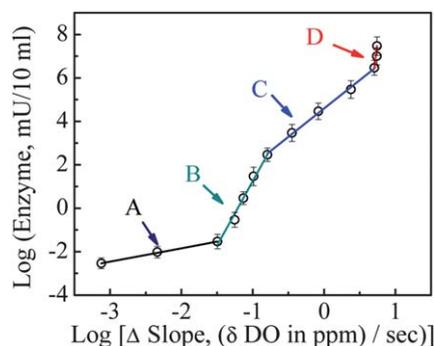


Fig. 3 Standard curve for catalase assay in the range of 2.93 μU to 29300 U 10 ml⁻¹ of reaction mixture using 34.095 mM H₂O₂ as substrate. Error bars represent standard deviation from a set of five.

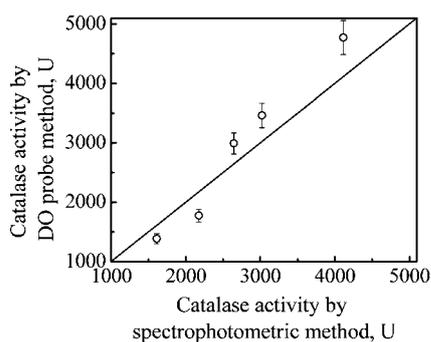


Fig. 4 Comparison between spectrophotometric and sensor methods for measurement of catalase activity in synthetic samples. Error bars represent standard deviation from a set of five, whereas the straight line represents ideal (unit slope) correlation between the methods compared.

nature of assay, such as between pathological testing and cellular level assays.

3.5. Assay of catalase in biological and real samples

The standard curve equations for the catalase assay obtained from the sensor were used as the basis for calculation of enzyme activity in biological samples such as tissue extracts of mice. Biological samples for assay of catalase were collected from Swiss albino mice. The amount of tissue homogenates obtained by sonication of liver, heart, brain and spleen samples has been listed in Table 1. It is known that catalase activities are highest in liver and erythrocytes, moderate in kidney, intermediate in lung and pancreas, and very low in heart and brain tissues.¹⁷ Therefore, in order to maintain the catalase concentrations within the detection limit of the spectrophotometric method, the stock of tissue homogenates was diluted with buffer, whereas no sample pre-dilution was necessary for sensor based estimation. Enzyme activity of catalase as calculated using both the methods was compared vis-à-vis (Fig. 5a–5f).

For the seven RBC samples analysed, the comparison data is shown in Fig. 5a. The levels of catalase activity in mice were in accordance with known values in wild-type mice (1000–5000 U ml⁻¹

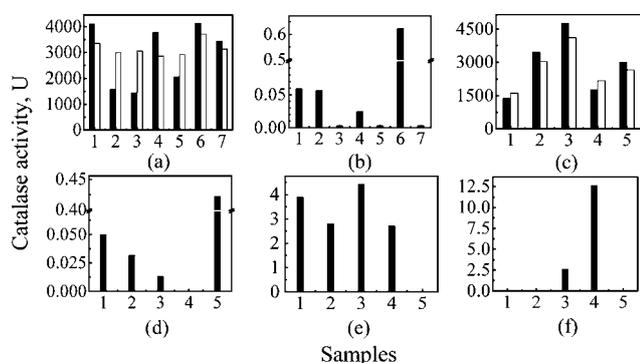


Fig. 5 Catalase activity in (a) RBC and (b) serum samples (U ml⁻¹ mice blood), (c) liver, (d) spleen, (e) brain and (f) heart tissue preparations U g⁻¹ wet weight of cells as determined by (■) sensor and (□) spectrophotometric method.

mouse blood).¹⁶ On the other hand, catalase activity could not be estimated by the spectrophotometric method in the case of serum samples, as its activity was too low.¹⁴ However, its enzyme activity could be determined using the sensor, owing to the high sensitivity and broad detection range of this method (Fig. 5b). The best comparison between both the methods was found while assaying catalase in liver samples (Fig. 5c), in which case enzyme activities as calculated by both methods were nearly identical. The enzyme activity found in this case was similar to the known levels of catalase activity (1000–7000 U g⁻¹ wet weight of tissue) in liver samples of wild-type mice.^{14,16} For spleen, brain and heart tissue homogenates, the spectrophotometric method was found unsuitable due to low catalase activity in these samples.¹⁴ Nevertheless, the sensor based assay could estimate the activity in these samples. The estimation data have been shown for spleen (Fig. 5d), brain (Fig. 5e) and heart (Fig. 5f) tissue samples.

From these results, it can be safely concluded that the sensor based assay method for catalase was suitable for determination of catalase activity in biological samples in a highly sensitive and accurate manner. Though this method was comparable to the existing spectrophotometric method over a particular range of catalase activities, it can be used as a separate and standard method for the assay of catalase over a broader detection range.

4. Conclusion

A novel approach for estimation of catalase activity in biological samples was developed using LabVIEW based virtual instrumentation. The assay was performed by monitoring an increase of DO concentration following the catalytic reaction of the enzyme with its substrate H₂O₂ using a Clark type DO electrode attached with op-amp based electronic circuit and computer interface. Perhaps in the first such report, a real time signal averaging technique was used in a biosensor measurement, which produced dramatic enhancement of assay sensitivity. Catalase activity in biological samples could be measured without pre-dilution and the method was not affected by interferences such as turbidity and viscosity of the sample. It was possible to obtain an assay sensitivity of 2.93 μU, compared to 0.5 U for the existing spectrophotometric method. Therefore, it is proposed that the method will find possible use in clinical assays, detection of pathogens, and evaluation of catalase activity in cell cultures, neurological samples, milk and other food products.

This work on the development of sensitive virtual instrumentation shall also be helpful to a wider community of researchers who either need enhanced protocols for amperometric/electrode based enzymatic assay at subcellular levels (using microelectrodes) or are aiming to develop instrumentation for such automated bio/chemical sensors.

5. Acknowledgements

Sandeep Kumar Jha was the recipient of a senior research fellowship from the Council of Scientific and Industrial Research, New Delhi, India. We are also thankful to the Department of Atomic Energy, Government of India and the Bhabha Atomic Research Centre, Mumbai, India for their infrastructure and financial support for this research.

6. References

- 1 L. Dominguez, J. F. Fernandez-Garayzabal, M. M. Blanco, V. Briones, J. A. Vazquez-Boland, J. Blanco and G. Suarez, *Z. Lebensm.-Unters. Forsch.*, 1990, **191**, 16–19.
- 2 Y. T. Han, Z. W. Han, G. Y. Yu, Y. J. Wang, R. Y. Cui and C. B. Wang, *Pharmacol. Res.*, 2004, **49**, 265–274.
- 3 T. L. Parkes, A. J. Elia, D. Dickinson, A. J. Hilliker, J. P. Phillips and G. L. Boulianne, *Nat. Genet.*, 1998, **19**, 171–174.
- 4 G. Cohen, D. Dembiec and J. Marcus, *Anal. Biochem.*, 1988, **174**, 331–336.
- 5 R. Artuch, C. Colome, C. Sierra, N. Brandi, N. Lambruschini, J. Campistol, D. Ugarte and M. A. Vilaseca, *Clin. Biochem.*, 2004, **37**, 198–203.
- 6 A. J. F. Griffiths, W. M. Gelbart, J. H. Miller and R. C. Lewontin, *Modern Genetic Analysis in An introduction to genetic analysis*, W. H. Freeman, New York, 1999.
- 7 S. Demirbilek, I. Gurses, N. Sezgin, A. Karaman and N. Gurbuz, *J. Pediatr. Surg.*, 2004, **39**, 57–62.
- 8 R. F. Beers, Jr. and I. W. Sizer, *J. Biol. Chem.*, 1952, **195**, 133–140.
- 9 G. Cohen, M. Kim and V. Ogwu, *J. Neurosci. Methods*, 1996, **67**, 53–56.
- 10 L. A. Del Rio, M. G. Ortega, A. L. Lopez and J. L. Gorge, *Anal. Biochem.*, 1977, **80**, 409–415.
- 11 L. Goth, *Clin. Chim. Acta*, 1991, **196**, 143–151.
- 12 L. H. Johansson and L. A. Borg, *Biochem. Biophys. Res. Commun.*, 2004, **315**, 502–508.
- 13 J. F. Huidobro, M. P. Sanchez, S. Muniategui and M. T. Sancho, *J. AOAC Int.*, 2005, **88**, 800–804.
- 14 R. E. Ganschow and R. T. Schimke, *J. Biol. Chem.*, **244**, 4649–4658.
- 15 L. H. Johansson and L. A. H. Borgi, *Anal. Biochem.*, 1988, **174**, 331–336.
- 16 H. Aebi, H. Suter and R. N. Feinstein, *Biochem. Genet.*, 1968, **2**, 245–251.
- 17 Y. J. Kang, Y. Chen and P. N. Epstein, *J. Biol. Chem.*, 1996, **271**, 12610–12616.
- 18 P. Vadgama and G. Davis, *Med. Lab. Sci.*, 1985, **42**, 333.
- 19 G. Rachmuth, K. Zhou, J. J. Monzon, H. Helble and C. S. Poon, *Sens. Actuators, B*, 2010, **149**, 170–176.
- 20 V. J. Sieben, C. F. A. Floquet, I. R. G. Ogilvie, M. C. Mowlem and H. Morgan, *Anal. Methods*, 2010, **2**, 484–491.
- 21 V. Thomsen, D. Schatzlein and D. Mercurio, *Spectroscopy*, 2003, **18**, 112–114.
- 22 J. Switala and P. C. Loewen, *Arch. Biochem. Biophys.*, 2002, **401**, 145–154.
- 23 M. A. Valdés García, P. Tuñón Blanco and A. Ivaska, *Electrochim. Acta*, 1998, **43**, 3533–3539.
- 24 M. Santucci, M. Portaccio, S. Rossi, U. Bencivenga, F. S. Gaeta and D. G. Mita, *Biosens. Bioelectron.*, 1999, **14**, 737–747.