Biosensors and Bioelectronics 24 (2009) 3487–3491

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Electrochemical assay of the nitrate and nitrite reductase activities of *Rhizobium japonicum*

J. Priscilla Salome, R. Amutha, P. Jagannathan, J.J.M. Josiah, Sheela Berchmans*, V. Yegnaraman

Central Electrochemical Research Institute, Electrodics and Electrocatalysis Division, Karaikudi 630006, Tamilnadu, India

ARTICLE INFO

Article history:
Received 18 February 2009
Received in revised form 25 April 2009
Accepted 30 April 2009
Available online 7 May 2009

Keywords:
Enzyme activity
Nitric oxide
Amperometry
Cyclic voltammetry
Biosensor
Rhizobium japonicum

ABSTRACT

This work describes an electrochemical method for the determination of the nitrate and nitrite reductase activities of *Rhizobium japonicum*. The advantage of the method lies in the use of whole cells for the analysis and we earlier developed this protocol for the assay of NO. The results obtained are comparable to the spectrophotometric Griess assay. As the method is based on electrochemical reduction, the commonly interfering biological components like ascorbic acid, uric acid, dopamine, etc., will not interfere with the analysis. This method can be extended to the fabrication of biosensors for nitrate and nitrite using the same principle.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Assimilation of CO_2 and inorganic nitrogen are the two most important metabolic processes carried out by plants. Adequate supply of carbon skeletons and photosynthetic energy for the assimilation of inorganic nitrogen via amino acids require accurate adjustment of these two pathways, a balance usually more easily maintained with nitrate than with ammonia. A special regulator should recognize both nitrate, the most important source of inorganic nitrogen at least in cultivated soils and the level of carbon source. It is known through centuries that the legumes enrich the soil by contributing nitrogen through symbiotic nitrogen fixation by *Rhizobium* species. Ever since the discovery of the presence of nitrate reductase in *Rhizobium japonicum*, there has been a considerable interest in the expression of various forms of this enzyme. Hence this system is of considerable interest for studying the nitrate reductase activity and nitrite reductase activity. Enzyme assays are usually made by incubating an enzyme with its substrate under conditions where ideally, the only biochemical reaction that occurs is the one that is measured. Generally such assays are carried out with cell free extracts in which the enzyme is readily accessible to the substrate. The preparation of cell free extracts is time consuming and tedious and correction has to be made for the fraction of cells that are broken during the assay. In such cases the enzyme activity is reported in terms of protein present in the preparation of the extract. Consequently errors increase. It is often convenient to estimate the enzyme activity in a sample of whole cells without extracting the cells. The free solubility of the substrate and the product and the ease of permeability across the cells facilitate assays using whole cells. Amperometric detection of lignin degrading peroxidase activities in culture filtrates from *Phanerochaete chrysosporium* has already been reported (Tang et al., 2005).

In this work we have demonstrated that the nitrate reductase and nitrite reductase enzyme activities of *R. japonicum* can be measured using whole cells. The nitrate reductase activity was evaluated by analyzing the nitrite produced by the nitrate reductase activity of the microbes when it is incubated with a sample of nitrate. Nitrite reductase activity is determined by incubating a sample of microorganisms with a sample of nitrite. The decrease in the concentration of nitrite is followed using our protocol.

Nitrite is widely recognized as nitrate's partner in environmental crime and in many respects can be considered to be the more dangerous of the two in terms of the potential influence of excessive concentrations on the health of humans and aquatic organisms. Nitrite is known to have primary role in methemoglobinemia (commonly characterized in human health terms as "blue baby" syndrome) and it has somewhat contentious implication in various cancers. Nitrite levels are routinely determined in quality control analyses of drinking, waste, marine and underground waters, among others (Icardo et al., 2001; Jodi and James, 2002; Jonathan et al., 1997). This has aroused an interest in developing new analytical

* Corresponding author. Tel.: +91 4565 227551/227559; fax: +91 4565 227779.
E-mail address: sheelaberchmans@yahoo.com (S. Berchmans).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.04.044

methods for determining nitrite in all types of samples. In this work we describe a new method for the estimation of nitrite. This protocol was earlier developed in our lab for NO assay (Mary Vergheese and Sheela, 2006). We demonstrate that nitrite can be indirectly analysed as NO due to its disproportionation reaction in concentrated sulphuric acid. Similar electrochemical detection of nitrate and nitrite based on NO assay has been carried out recently in acidic iodide bath (Boo et al., 2007). Recently, multiple physiologic studies have surprisingly revealed that nitrite represents a biological reservoir of NO that can regulate hypoxic vasodilation, cellular respiration, and signaling (Gladwin and Kim-Shapiro, 2008). The scientific literature is littered with protocols for the detection of both nitrate and nitrite but a cursory examination will reveal that the majority employs nitrite as the primary detection protagonist (Sah, 1994; Payne, 1991; Moorcroft et al., 2001; Fox, 1985; Davis et al., 2000).

The most widely used is the Griess reaction, in which nitrite is chemically transformed into a coloured diazo compound by reaction with sulfanilamide and *N*-(1-naphthyl) ethylenediamine (Griess, 1879). Nitrate can be measured by this reaction following chemical reduction to nitrite by Cd-coated particles or via enzymatic reduction by nitrate reductase (Cortas and Wakid, 1990; Giovannoni et al., 1997; Syrett, 1973). This method has adequate sensitivity to monitor biological systems; however, the assays take a relatively long time to perform, because the reaction consists of two steps: the reduction of nitrate to nitrite and then the azo derivatization and spectrophotometric detection of nitrite. The protocol described in this work is perhaps one of the pioneering electrochemical methods reported for nitrate reductase and nitrite reductase enzyme activities measurement in whole cells. The method described here also opens up the possibility of fabrication of nitrite and nitrate biosensor based on the same principle.

2. Experimental

2.1. Reagents

The following chemicals were used as received for the experiments: KH_2PO_4 (Merck), $MgSO_4$ (Hi Media), NaCl (Hi Media), $CaCO_3$ (HiMedia), yeast extract (HiMedia), malt extract (HiMedia), sucrose (SRL), $CaSO_4$ (SRL), mannitol (Loba Chemie), maltose (Loba Chemie), sulfanilamide (Acros Organics), and *N*-napthyl ethylenediamine dihydrogen chloride (Acros Organics).

2.2. Subculturing of R. japonicum

Rhizobium japonicum (NCIM2747) was subcultured using the following culture medium: Lochhead's medium which consists of KH_2PO_4, 0.4 g; $MgSO_4{\cdot}7H_2O$, 80 mg; NaCl, 80 mg; $CaSO_4$, 50 mg; $CaCO_3$, 50 mg; sucrose, 10 g; mannitol, 4.0 g; maltose, 2.0 g; yeast extract, 1.0 g; malt extract, 0.24 g; distilled water, 1000 ml. The cells were harvested after 48 h and separated by refrigerated centrifuging at 10,000 rpm and suspended in phosphate buffer (pH 7.0).

2.3. Estimation of nitrite by Griess assay

The absorbance of the intense purple coloured diazo compound formed on the addition of the reagents sulfanilamide in acid solution and *N*-naphthyl ethylene diamine was analysed at 540 nm. Griess assay was carried out using spectrophotometer (Varian, Cary5000).

2.4. Estimation of nitrite by electrochemical assay

The glassy carbon electrode was polished to a mirror finish with fine emery paper using alumina paste and then sonicated in Millipore water for 5 min. The surface purity was checked by running a cyclic voltammogram with 1 mM ferrocyanide solution in 0.5 M of sulphuric acid, which exhibited reversible features. Then the electrode was modified with ferrocene solution (500 μl of 1% Nafion mixed with 100 μl of 20 mg/ml ethanol solution of ferrocene).

The electrochemical assay was carried out using PARSTAT 2263 using a three electrode arrangement with ferrocene-modified glassy carbon as the working electrode, Pt counter electrode and $Hg/Hg_2SO_4/0.5\,M\ H_2SO_4$ reference electrode. This reference electrode has a potential of 0.680 V vs. NHE. The electrochemical reduction of nitrite under acidic conditions is monitored. The potential of the working electrode was fixed at −0.4 V vs. Hg/Hg_2SO_4 and the amperometric estimations were carried out. 0.5 M sulphuric acid was used as the supporting electrolyte and the assay was carried out under ambient conditions.

2.5. Preparation of sample for nitrate reductase activity

1 ml of the culture suspension was mixed with 0.3 ml of potassium nitrate (0.1 M stock) and 1 ml of phosphate buffer (pH 7.0) for 3 h. After 3 h the sample was centrifuged at 10,000 rpm for 10 min and the supernatant solution was analysed by Griess assay and electrochemical assay.

2.6. Preparation of sample for nitrite reductase activity

1 ml of the culture solution was mixed with 0.3 ml of nitrite solution (10 mM stock) and 1 ml of phosphate buffer (pH 7.0) solution for 3 h and then the sample was centrifuged at 10,000 rpm for 10 min and the supernatant solution was analysed by Griess assay and electrochemical assay.

2.7. Epifluorscence micrographs

Epifluorescence imaging of the microorganisms was carried out using the instrument Nikon Eclipse E200.

3. Results and discussion

3.1. Harvesting and characterization of R. japonicum

The cells were grown under different conditions. Three different nitrogen sources are used for the preparation of cultures, viz., yeast extract, glutamate and nitrate. The cells were cultured in aerobic and anaerobic conditions. In all the conditions the growth was observed and the cultures were prepared under these six different conditions (three different nitrogen sources under aerobic and anaerobic conditions). The cells were harvested after 48 h, centrifuged at 10,000 rpm and suspended in 5 ml of phosphate buffer. Wet weight of the cells was noted during all the trials.

The morphology of the cells can be seen from the epifluroscence micrograph (see supporting information). The rod-like morphology is revealed by the epifluroscence micrograph. The culture sample positively responded to all the characteristic biochemical tests.

3.2. Qualitative test for the presence of nitrate and nitrite reductase activity in R. japonicum

The presence of the enzyme activity (nitrate and nitrite reductase activity) in *R. japonicum* was evaluated by the following procedure using Griess assay.

Four samples were prepared for the analysis. Samples 1 and 2 consisted of 0.2 ml of cell suspension + 0.2 ml nitrite (10 mM stock). Immediately on mixing the reagents sample 1 was centrifuged and the cells are discarded and the supernatant was treated with Griess reagent and it gives rise to a pink colour. Sample 2 was allowed

to incubate for 1 h and then the sample was centrifuged and the supernatant was treated with Griess reagents. The intensity of the pink colour was reduced compared to sample 1. This test shows that nitrite is consumed by the cells due to the nitrite reductase activity. Hence intensity of pink colour decreases as a result of consumption of nitrite.

Samples 3 and 4 consisted of 0.2 ml of cell suspension + 0.2 ml of nitrate solution (0.1 M stock) and 1 ml succinate (stock = 0.25 M). Succinate is required only in the case of cultures grown with yeast extract as the nitrogen source. Sample 3 was centrifuged immediately after mixing the analyte and the supernatant was treated with Griess reagent. No pink colour was developed. Sample 4 was allowed to incubate for 1 h and then it was centrifuged to remove the cells and the supernatant was treated with Griess reagent. A pink colour was produced which indicates that nitrite is produced by the nitrate reductase activity of the cells.

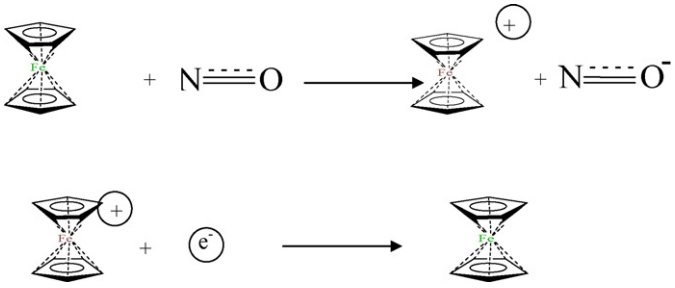
3.3. Principle of electrochemical determination

The assay is based on the following disproportionation reaction (Sun et al., 1996)

$$H^+ + 2NO + NO_3^- + H_2O \Leftrightarrow 3HNO_2, \quad K = 1.1 \times 10^{20}$$

HNO_2 is the reactive form of nitrite at pH < 3 and nitrite is the reactive form at pH > 3. The dissociation constant of HNO_2 is 5.1×10^{-4} and its $pK_a = 3.3$. Hence in acidic conditions nitrite is indirectly determined as NO formed during the disproportionation reaction. Hence this work demonstrates how our novel protocol developed earlier for the analysis of NO can be extended to the determination of nitrate and nitrite reductase activity.

The mechanism of electrochemical assay of NO is proposed as follows:



Ferrocene undergoes one electron reversible charge transfer reaction. The chemical reduction of NO coupled to the Fc/Fc^+ charge transfer is the basis of NO estimation reported by us earlier. In this work we estimate nitrite ions in acidic medium indirectly as NO and use the same principle to detect the nitrate reductase and nitrite reductase activity of the microorganism *R. japonicum* (Fig. 1).

Nitrate reductase

$$NO_3^- + AH_2(\text{electron donor}) \rightarrow NO_2^- + A + H_2O(\text{nitrite formation is the target of analysis})$$

Nitrite reductase

$$NO_2^- \rightarrow NH_4^+(\text{nitrite disappearance is the target of analysis})$$

The assay of nitrate reductase activity is possible, if along with nitrate, an electron donor is also added (viz., succinate, malate, fumarate, etc.) (Siva Raju et al., 1997; Stohr and Ullrich, 1997; Phillips et al., 1973; Streeter and Devine, 1983; Salsac et al., 1987). The electron donor maintained a supply of NADH in the cell. In this work we found out that under certain growth conditions, addition of electron donor is not required (see supporting information, Table 1).

3.4. Electrochemical detection of nitrate and nitrite reductase activities

Fig. 2 shows the typical response of the glassy carbon electrode modified with ferrocene as explained in Section 2. The ferrocene-modified electrode exhibits near reversible features. The modification was repeated every time when a calibration is required. The peak characteristics for ferrocene-modified electrode are given in Table 1 (average for 10 trials is given).

Fig. 3 depicts the cyclic voltammograms representing the electrocatalytic reduction of NO by ferrocene. With increase in the concentration of nitrite the catalytic current increases proportionately. This forms the basis of nitrite estimation. Amperometric experiments were recorded at the reduction potential of NO and the results are used for the estimation of enzyme activity.

Fig. 4 depicts the typical amperometric curve obtained with standard nitrite solutions and this standard curve was used for constructing the calibration curve for the estimation of nitrite grown with yeast as nitrogen source. Similarly twelve amperometric experiments were run separately for standard nitrite additions and used for the construction of calibration graph for the estimation of nitrate reductase and nitrite reductase activities of the enzymes under different growth conditions. The current proportionately increases with increase in the concentration of nitrite in the amperometric curves.

Calibration graph was plotted for each estimation and the typical calibration graph for four cases is presented in Fig. 5 for four different growth conditions as given in the figure. All the plots correspond to aerobic conditions with different nitrogen sources as given below:

Glutamate-nitrite—Glutamate: nitrogen source; analyte: nitrite.
Glutamate-nitrate—Glutamate: nitrogen source; analyte: nitrate.
Yeast-nitrite—yeast: nitrogen source; analyte: nitrite.
Yeast-nitrate—yeast: nitrogen source; analyte: nitrate.

The calibration graphs exhibited very good linearity. Liner fit was performed and the average features of the linear fit are as follows—correlation coefficient: 0.99; SD: 0.05; LOD: 20 μM; sensitivity: 6 μA/mM. The electrode can be used for about 3–4 assays and the reproducibility of the sensor is within 1%. The nitrite formed or consumed is a function of the nitrate reductase and nitrite reductase activities. Different growth conditions give rise to different types of nitrite and nitrate reductase activities. Hence the electrochemical response is different for different growth conditions.

Amperometric curve was obtained for the estimation of the nitrate and nitrite reductase activities under different growth conditions (Fig. 2 Supporting information). From the amperometric curves, the current corresponding to nitrite reduction is obtained and the estimate of the enzyme activity is made from the calibration graph. From the reduction current and using the corresponding calibration graph, the activities are calculated and presented in supporting information (Table 2). Similar experiments were carried out with Griess assay using spectrophotometer and the results are within 2% error.

3.5. Ferrocene based sensing of anions

The ferrocene based anion sensing is known for phosphate ions, adenosine monophosphate (AMP) and adenosine triphosphate (ATP) where the anodic as well as cathodic current decreases in the presence of the anions. This has been shown with the help of different generations of poly(propylene imine) dendrimers functionalized with ferrocenyl urea and pentamethyl amidoferrocenyl metallo dendrimers. This type of anion sensing has been demonstrated only in aprotic media (Daniel et al., 2003; Ruiz et al., 2003;

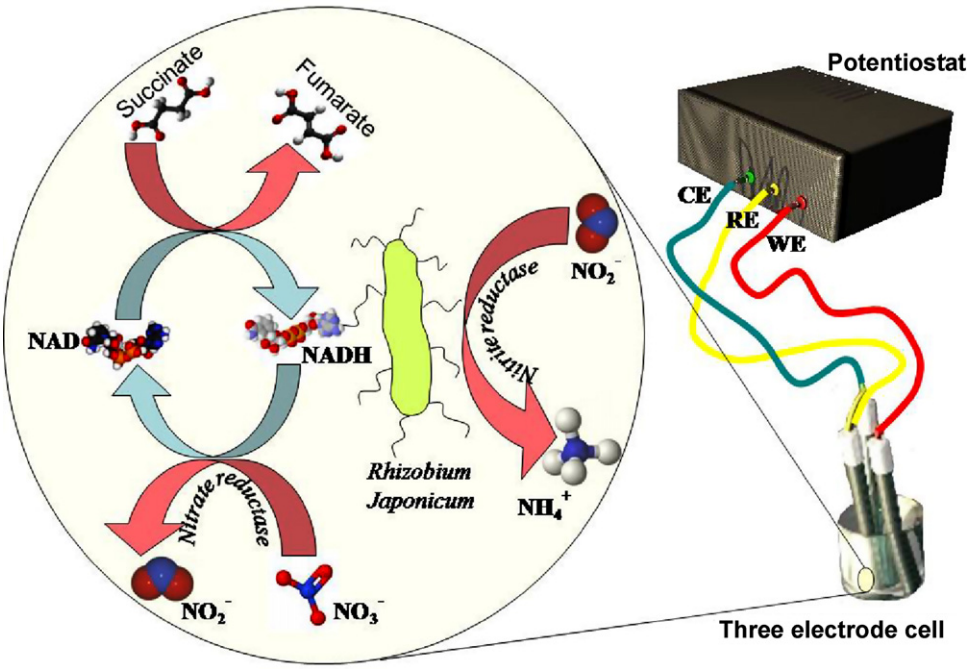


Fig. 1. Mechanism of electrochemical detection.

Table 1
Peak characteristics of the ferrocene-modified electrode.

▽Ep (V)	FWHM (V)	Ep_a (V)	Ep_c (V)	Anodic coverage (mol/cm^2)	Cathodic coverage (mol/cm^2)	Ip_a/Ip_c
0.083	0.09	−0.307	−0.390	1.75×10^{-8}	1.85×10^{-8}	1.0

Alonso et al., 2002). Surface pre-organisation within robust amido-ferrocene self-assembled monolayers have been exploited in the selective electrochemical sensing of anions in both organic and aqueous media (Beer et al., 2002). The interaction between the amide groups and the anions is found to be responsible for the decrease in the oxidation and reduction current of Fc/Fc^+ couple. In our work the chemical reduction of NO by ferrocene in acidic medium is coupled with the electrochemical reduction of ferrocene and selectivity is achieved.

3.6. Addition of electron donors for the determination of nitrate reductase activity

It has been described in the literature that the nitrate reductase activity depends upon electron donors like succinate, pyruvate, malate, etc., and they are required for maintaining a supply of NADH in the cells. We have studied the enzyme activity in six different situations. The addition of electron donor is required in certain cases only (Table 1 Supporting information).

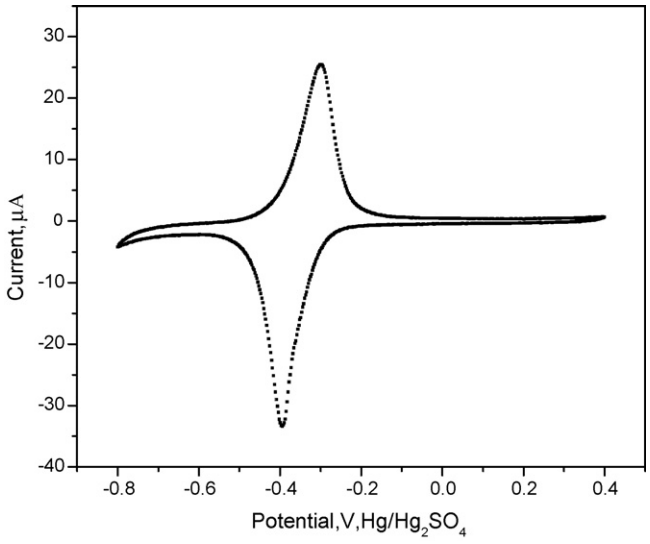


Fig. 2. Typical cyclic voltammetric response of the ferrocene-modified glassy carbon electrode in 0.5 M sulphuric acid; scan rate = 50 mV/s.

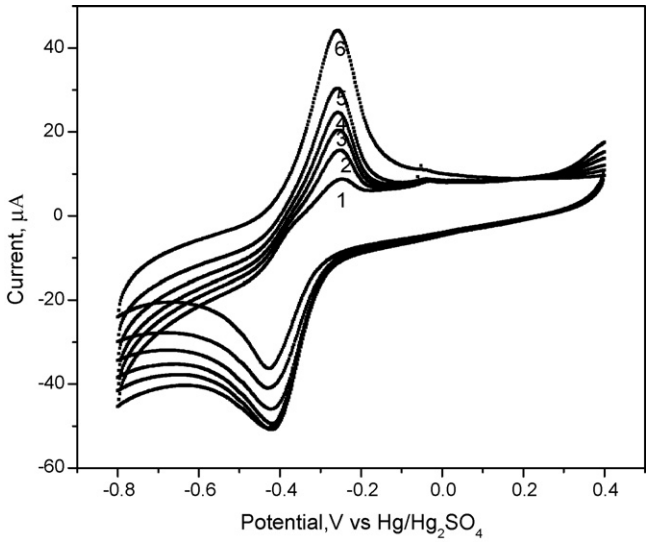


Fig. 3. Cyclic voltammograms representing electrocatalysis of nitrite for different additions of nitrite: (1) 0.476, (2) 0.385, (3) 0.291, (4) 0.196, (5) 0.099, and (6) 0 mM, in 0.5 M sulphuric acid; scan rate = 50 mV/s.

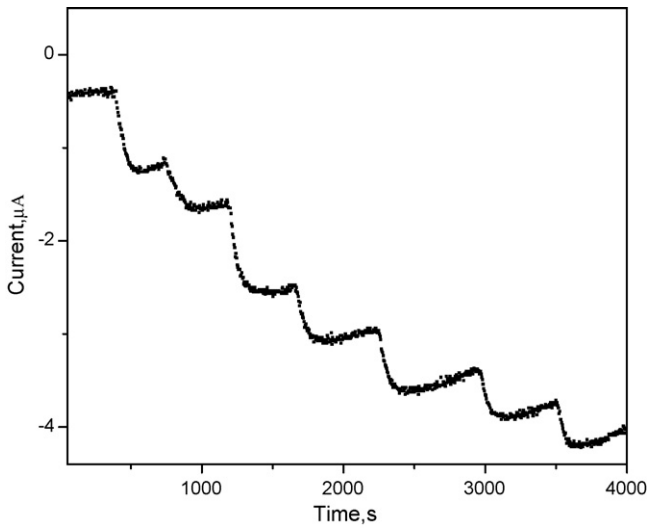


Fig. 4. Typical amperometric responses for the standard additions of nitrite. Each addition corresponds to 50 μl of 10 mM stock. Potential applied −0.4 V vs. Hg/Hg_2SO_4.

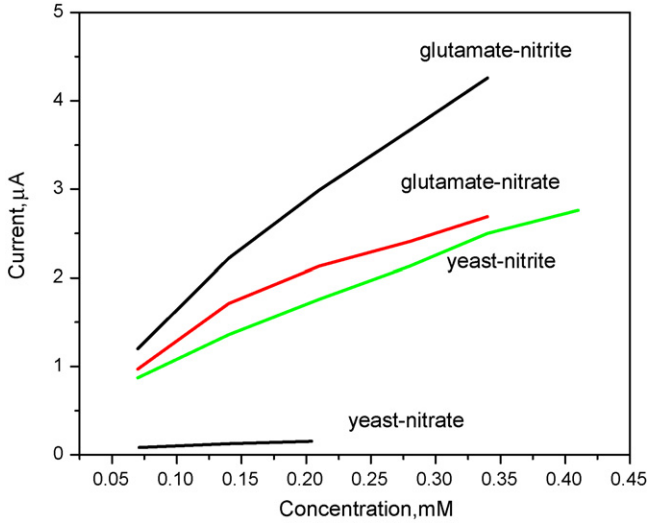


Fig. 5. Calibration plot obtained for four typical analyses.

3.7. Advantages of electrochemical assay

For the determination of enzyme activities two different procedures are normally used (Kiang et al., 1979). Nitrate reductase activity is measured in terms of nitrite produced, using Griess assay in the presence of NADH. Nitrite reductase activity is measured in terms of consumption of nitrite where reduced methyl viologen acts as the electron donor. In the case of our electrochemical assay, the same modified electrode can be used for the determination of the enzyme activities under the same applied potential. The electrochemical method is highly reproducible and free from interferences as the detection is based on reduction.

In the case of Griess assay, the assay should be made as soon as the colour is developed. Otherwise, the procedure leads to erratic results. Amperometric detectors are portable and ideal for field measurements. Attempts have also been made for measuring the nitrite reductase activity in leaves of wheat plant grown in our laboratory. The plant is watered with dilute solutions (μM) of potassium nitrate and nitrite reductase activity was estimated by Griess assay and electrochemical assay. *Rhizobium* sp. was isolated from the root nodules of groundnut plants collected from nearby fields and the nitrate and nitrite reductase activities of the microorganisms could be estimated using our protocol. Further the electrochemical assay has been carried out with PARSTAT 2263 which is a portable model and is ideal for field measurements. The investigations with wheat leaves and *Rhizobium* sp. isolated from the root nodules of the groundnut plants indicate the viability of our experimental protocol under field conditions. The analytical protocol described in this method can be extended to the fabrication of enzymatic biosensors for nitrate and nitrite towards which the work is in progress.

4. Conclusions

In this work we have demonstrated how our NO sensing protocol recently developed in our laboratory can be extended to the analysis of nitrate and nitrite reductase activities of *R. japonicum*. This method is found to be advantageous compared to the conventional methods. It has also been shown that whole cells can be used for estimation of the enzymatic activity. The detection of nitrate reductase activity is based on the nitrite produced and nitrite reductase activity is based on the nitrite consumed. This method is being extended to develop nitrate and nitrite enzyme based biosensors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.04.044.

References

Alonso, B., Casado, C.M., Cuadrado, I., Morána, M., Kaifer, A.E., 2002. Chem. Commun., 1778–1779.
Beer, P.D., Davis, J.J., Drillsma-Milgrom, D.A., Szemes, F., 2002. Chem. Commun., 1716–1717.
Boo, Y.C., Tressel, S.L., Job, H., 2007. Nitric Oxide 16, 306–312.
Cortas, N.K., Wakid, N.W., 1990. Clin. Chem. 36, 1440–1443.
Daniel, M., Ruiz, J., Blais, J., Daro, N., Astruc, D., 2003. Chem. Eur. J. 9, 4371–4379.
Davis, J., Moorcroft, M.J., Wilkins, S.J., Compton, R.G., Cardosi, M.F., 2000. Analyst 125, 737–742.
Fox, J.B., 1985. CRC Crit. Rev. Anal. Chem. 15, 283.
Gladwin, M.T., Kim-Shapiro, D.B., 2008. Blood 112, 2636–2647.
Griess, J.P., 1879. Ber. Dtsch. Chem. Ges. 12, 426–429.
Giovannoni, G., Land, J.M., Keir, G., Thompson, E.J., Heales, S.J.R., 1997. Ann. Clin. Biochem. 34, 193–198.
Icardo, M.C., Calatayud, J.M., Mateo, J.V.G., 2001. Analyst 126, 1423–1427.
Jodi, D., James, D., 2002. J. Environ. Monit. 4, 465–471.
Jonathan, W.A., David, J.R., David, A.R., 1997. Analyst 122, 77–80.
Kiang, C., Kuan, S.S., Guilbault, G.G., 1979. Anal. Chem. 50, 1319.
Mary Vergheese, T., Sheela, B., 2006. Electrochim. Acta 52, 567–574.
Moorcroft, M.J., Davis, J., Compton, R.G., 2001. Talanta 54, 785.
Payne, W.J., 1991. Forest Ecol. Manage. 44, 5.
Phillips, D.A., Daniel, R.M., Appleby, C.A., Evans, H.J., 1973. Plant Physiol. 51, 136–138.
Ruiz, J., Medel, M.J.R., Daniel, M., Blaisb, J., Astruc, D., 2003. Chem. Commun., 464–465.
Salsac, L., Chaillou, S., Morot-Gaudry, J.F., Lesaint, C., Jolivet, E., 1987. Plant Physiol. Biochem. 25, 805–812.
Sah, R.N., 1994. Commun. Soil Sci. Plant Anal. 25, 2841.
Siva Raju, K., Sharma, N.D., Lodha, M.L., 1997. FEMS Microbiol. Lett. 151, 17–21.
Stohr, C., Ullrich, W.R., 1997. Planta 203, 129–132.
Streeter, J.G., Devine, P.J., 1983. Appl. Environ. Microbiol., 521–524.
Sun, W., Liu, H., Kong, J., Xie, G., Deng, J., 1996. J. Electronal. Chem. 408, 261.
Syrett, P.J., 1973. New Phytol. 72, 37–46.
Tang, L., Zenga, G., Wang, H., Sun, G., Hunga, D., 2005. Enzyme Microb. Technol. 36, 960–966.