

# Research & Reviews : Journal of Ecology and Environmental Sciences

## Construction of Nitrate Reductase Biosensor using Lab -on -Paper Technology and its Application in Nitrate level Management

Pankti Rajal, Meghashree Deshmukh, Leena Phadke  
Department of Microbiology, Ramnarain Ruia College, Matunga, India.

### Research Article

Received date: 15/08/ 2015

Accepted date: 17/12/ 2015

Published date: 24/12/2015

#### \*For Correspondence

Pankti Rajal, Department of Microbiology,  
Ramnarain Ruia College, Matunga, India.

E-mail: pankti.rajal@gmail.com

**Keywords:** Nitrate reductase biosensor,  
Potentiostat, Screen printed electrodes.

**Abbreviations:** Nitrate Reductase (NR), N-(1-naphthyl)-ethylene diamine hydrochloride (NEED), hydrogen peroxide ( $H_2O_2$ ), Screen printed Electrodes (SPE), micro ampere ( $\mu A$ ).

#### ABSTRACT

Amperometric screen printed nitrate reductase biosensor has been developed to detect nitrate ions in aqueous environmental samples. The biosensor was designed by immobilizing the enzyme using glutaraldehyde with coupling agents Azure A and  $H_2O_2$  as mediators on screen printed carbon electrodes. The biosensor developed was able to detect nitrate ions in different environmental samples up to 20ppm which is within the limit as suggested by European Union standards, representing cheap, rapid and applicable tool for environmental nitrate analysis.

### INTRODUCTION

Everything that survives requires nitrogen. Nitrogen is available in various chemical forms in the ecosystem by global nitrogen cycle. However, the balance created by nitrogen cycle is disturbed by human intervention through globalization and urbanization. It includes activities like uncontrolled use of chemical fertilizers, combustion of fossil fuels, sewage effluents, etc. These alterations in the Nitrogen cycle will pose a serious threat to human welfare significantly rising the nitrate concentration in land and water causing acidification, nutrient imbalance, loss of biodiversity and serious life threatening health problems which include Methemoglobinemia/blue baby syndrome and gastric ulcers<sup>[1]</sup>. Accumulated nitrate in the environment is converted to nitrite, a hazardous substance, by the action of various microbes present in the environment and also by the residing oral flora in the mouth of the individual. Hence, as a control measure, Union European Standard defines 50ppm as the limit of nitrate content in the environmental samples<sup>[2][3]</sup>. The techniques used to detect the levels of nitrate are assays based on spectroscopic, colorimetric or chromatographic methods which are cumbersome, laborious and expensive method requiring trained laboratory person<sup>[4-6]</sup>.

Thus, the main objective of this research is to design a biosensor that is cheap, user friendly, rapid and accurate to determine the nitrate levels in the environment within minutes. Biosensors are based on the principle that consists of a biological recognition system often called a bio receptor and a transducer. The interaction of an analyte with the bio receptor is designated to produce an effect measured by the transducer which converts the electrochemical signal into electrical signal<sup>[4]</sup>.

The principle underlying this biosensor is based on the catalytic activity of nitrate reductase enzyme (bioreceptor) which reacts with nitrate ions (analyte) present in the sample. The electron transfer takes place, signal transduction is achieved through electrochemical approaches which is detected amperometrically using Potentiostat.

Fabrication and designing of biosensors has been achieved by soft lithography, lab on chip (L-O-C), lab on paper (L-O-P),

etc. Among these techniques, lab on paper (L-O-P) is the cheapest and the easiest technology for hydrophilic liquids because of inherent properties of paper. Advantages of using this technology are – instrumentation cost is reduced considerably; cheap production and reproducibility, disposable, no availability issues of the substrate (Paper), etc.<sup>[2][3]</sup>.

In this research, biosensor has been fabricated using lab on paper technology for environmental nitrate level monitoring. Whatmann filter paper number 1 was used as the substrate. Screen printed electrodes (SPE) were designed for amperometric detection of nitrate. The nitrate in to the environmental sample gets converted in to nitrite by nitrate reductase enzyme immobilized on to the biosensor, releasing electrons, which is detected amperometrically.

In particular, in amperometric approach it was found that the biosensor chip devised was cheap, rapid, easy to use, easy to manufacture, reproducible but could not be used on site due to the limitation of usage of potentiostat. However, the software used for potentiostatic method could be developed on to a small device that is handy, sensitive and selective<sup>[4]</sup>.

## MATERIALS AND METHODS

### Chemicals

Nitrate reductase enzyme extracted from the organism *Providencia stuartii* was used. Glutaraldehyde,  $H_2O_2$ , Azure A, Potassium nitrate, Bovine serum Albumin taken from LoBA chemicals (India) was used. Electrochemical experiments were carried out in Sodium phosphate buffer (0.1M; pH6.8). Enzyme solution was prepared in Tris-HCl buffer (pH 7.0). Conductive carbon and silver adhesive paste purchased from Alpha Essar (USA) was used. All the chemicals used were of analytical or better grade.

### Apparatus

- Steps for preparing biosensor:

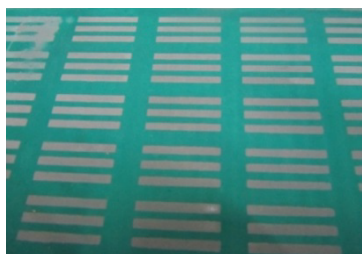
#### 1. Patterning of template for fabrication of biosensor

Rectangular blocks of 19 x 2 mm dimension were made using Corel Draw Graphic suite X5.

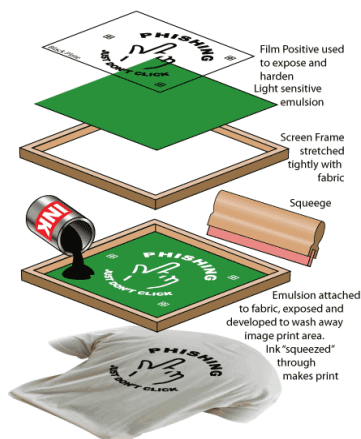
The design is printed on to the transparency film. This is used as a positive film for screen printing.

#### 2. Screen printing template

A light sensitive PVA solution was forced through the rayon mesh screen by a rubber squeegee and was allowed to dry. Next, a positive film (printed biosensor pattern on transparency) was exposed to tube light for 15 – 20 minutes, for hardening of light sensitive emulsion. Rinsed with water and was allowed to dry. The screen was ready to use for patterning of biosensor on to different substrates forming a stencil for printing<sup>[1][7]</sup>. Refer **Figures 1 and 2**.



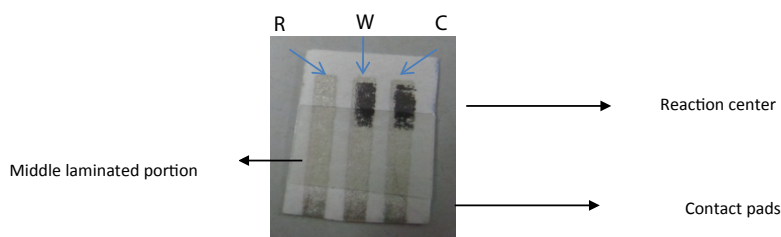
**Figure 1.** Screen Printing Template.



**Figure 2.** Shows the schematic diagram for screen printing protocol.

#### 3. Screen printing of biosensor

The biosensor design comprised of 3 electrodes – Reference (R – Silver electrode), Working (W – Carbon electrode with Enzyme) and Counter (C – Carbon electrode as blank) electrode using screen printing as shown in **Figure 3**.



**Figure 3.** Screen Printed nitrate reductase Biosensor on to Whatmann filter paper.

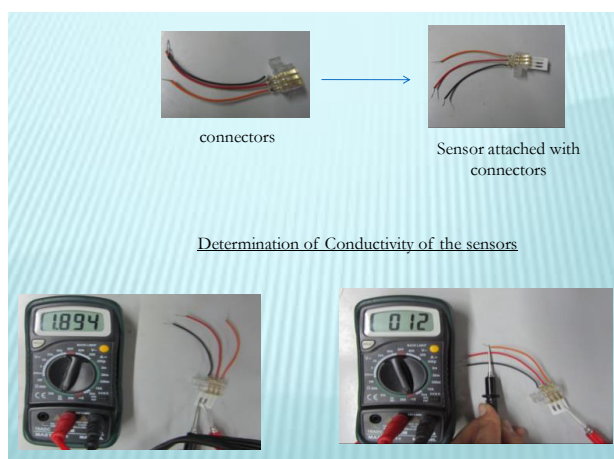
Screen printing involved multiple steps. First, Screen print template of the desired biosensor design was made. A layer of silver ink was deposited to define the conducting track and reference electrode on to Whatmann filter paper number 1 (125 mm) using the screen printing template. After it is dried, consecutive layer of carbon ink was placed only at the reaction centre of working and counter electrodes as seen in **Figure 3** and allowed to dry. Later, the middle portion of it was laminated using transparency, leaving the contact pads unlaminated <sup>[2][4,8]</sup>. Electrochemical measurements were done using Potentiostat Palm Sens (Palm Instruments BV, Netherland).

### Preparation of silver chloride electrode

After Reference electrode denoted as R in **Figure 3**, is completely dried, 5  $\mu\text{l}$  of 0.1 M  $\text{FeCl}_3$  solution was added and allowed to react for 5–10 minutes and later was wiped off using a cloth or filter paper <sup>[2]</sup>. This makes silver chloride electrode. This step is necessary because silver chloride reference electrode serves better than silver coating alone during potentiostatic analysis <sup>[1]</sup>.

### Determination of conductance of the biosensor

It was done using a multimeter. The conductivity of Reference, Working and Counter electrode was checked <sup>[5]</sup> in **Figure 4**.



**Figure 4.** Conductivity test for Biosensors.

### Pre-treatment of electrodes

The potential of + 2V (versus Reference Electrode) for 3 minutes was applied by dipping the electrodes in 5 ml of 0.25 M acetate buffer, containing 10 mM KCl (pH 4.8–5), stirring it continuously. Potential was applied using power pack <sup>[5,9-12]</sup>.

### Immobilization of enzyme on to the biosensor

5 mM Azure A and 5 mM  $\text{H}_2\text{O}_2$  were added in 0.1 M Sodium phosphate buffer pH 6.8, 2  $\mu\text{l}$  of this solution was added on to the working electrode of screen printed biosensor. It was air dried. Successively, the enzymatic membrane was prepared directly by dropping 5  $\mu\text{l}$  of mixture of BSA (0.1 mg/ $\mu\text{l}$ ): glutaraldehyde (1%): enzyme (0.2 mg/ $\mu\text{l}$ ) in the ratio 20:10:5 on working electrode. SPE was stored at 28°C for 1 hr and then successively at 4°C overnight <sup>[2][4]</sup>.

### Sample processing for nitrate detection

Water samples are directly subjected to the biosensor for nitrate analysis. For soil samples: 0.1 g of soil was mixed with 10 ml D/W, it was vortexed, kept aside for the particles to settle down. The supernatant was taken and used as the soil sample. These were used for both potentiostatic analysis and conventional enzyme assay.

### Potentiostatic analysis

The reaction centre of Screen printed electrodes was dipped in nitrate standards at different concentrations, soil sample and in laboratory D/W respectively. The current corresponding to the reduction of azure A and  $\text{H}_2\text{O}_2$  at -600mV was measured by potentiostat as shown in **Figure 5** <sup>[1][2]</sup>. Measured current was analysed using Prim chem software installed on to the connecting computer. The current is generated due to the electrons released as a result of reduction of nitrate by nitrate reductase enzyme.

Thus, the current corresponds to the amount of nitrate in the respective solution. Hence the amount of nitrate in the given soil sample and laboratory D/W can be calculated by comparing it with standard nitrate solutions. The software measures the current produced in  $\mu\text{A}$ <sup>[10]</sup>.



Figure 5a. Potentiostat.



Figure 5b. Setup of biosensor for potentiostatic analysis.

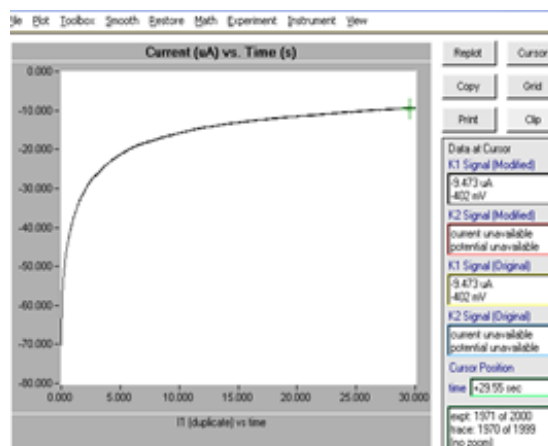


Figure 5c. Representative graph generated by Primchem Software analysing amount of current produced.

#### Conventional colorimetric enzyme assay

0.5ml each of varying concentrations of potassium nitrate solution ranging from 10 ppm to 100 ppm was mixed with 0.5ml of Nitrate reductase enzyme (specific activity – 75 units/mg) and allowed to react for 10 minutes at room temperature. Later, 0.5 ml of 1% w/v sulphanilamide in 25% HCl and 0.5 ml of NEED solution was added. After 10 minutes, it was read at 540 nm. Soil and water samples were used to determine the level of nitrate in it<sup>[6]</sup>.

## RESULTS AND DISCUSSION

#### Potentiostatic analysis using biosensor for nitrate detection

The current corresponding to the reduction of  $\text{H}_2\text{O}_2$  is ideally measured at -800 mV and Azure A at -400 mV by potentiostat. But, since both Azure A and  $\text{H}_2\text{O}_2$  were used in immobilization of enzyme onto the biosensor, the current for nitrate reductase biosensor was measured at -600 mV for 30 seconds. With the help of Prim chem software, the current generated as a result of reaction between nitrate and nitrate reductase enzyme is measured. It measures the current in  $\mu\text{A}$  within the period of 30 seconds and provides the output current which is considered for analysis as shown in **Tables 1 and 2**.

Table 1. Output of the Data generated through Prim Chem. Software.

Standard Nitrate Solution (ppm)	Current Generated Through Potentiostat ( $\mu\text{A}$ )
10	0.00
20	-59.28
40	-9.473
60	-5.566

80	-3.086
100	-2.930
Soil sample	-8.203
Lab D/W	-9.863

**Table 2.** Comparison of the Conventional Method and the Potentiostatic Method.

Sample tested for presence of nitrate	Amount of nitrate detected in the sample (ppm) By Potentiostatic method (biosensor)	Amount of nitrate detected in the sample (ppm) by Conventional method (enzyme assay)
Soil sample	49.75	50
Laboratory D/W	28.5	24

The current generation has negative values indicating reduction taking place at biosensor reaction centre with release of electrons. Taking the negative scale into consideration, as the concentration of nitrate increases, more current will be generated which will be proportional to the concentration of nitrite formed. Hence, as the concentration of nitrate is increased from 10 to 100 ppm, progressively more current is being detected from  $-59.28 \mu\text{A}$  to  $-9.863 \mu\text{A}$  (on the negative scale, bigger number indicates less value). On comparison of nitrate concentration in the samples with the standard nitrate solutions, it was found that the laboratory water sample and soil sample contain 28.5 ppm and 49.75 ppm of nitrate respectively<sup>[1]</sup>.

### Comparison between the conventional method (enzyme assay) and potentiostatic method for determination of nitrate content in different samples

The amount of nitrate present in the soil sample and laboratory D/W was detected by both conventional and potentiometric method. It was seen that the results obtained by both these methods are similar. So, the new biosensor designed is almost accurate to the conventional method<sup>[1]</sup>.

However, the biosensor designed has many advantages than the conventional method:

1. The time taken for nitrate detection by the biosensor is only 30 seconds whereas the conventional technique requires certain time for performing the Assay for nitrate detection. So, the biosensor prepared helps in rapid analysis of nitrates in the sample.
2. About 5  $\mu\text{l}$  of reagents are required for potentiometric detection method rather than the conventional method which utilizes about 2 ml of reagents for detection of single sample, making it economical.
3. More glassware is used while performing the conventional method. However, for potentiometric detection no glassware is required.
4. All these factors reduce the cost of detection of nitrate in the samples using biosensors<sup>[1]</sup>.

## CONCLUSION

In this study, an amperometric NR biosensor was developed in order to investigate the concentration of nitrate in the environmental samples which was accurate in comparison with the conventional colorimetric assay. Thus, it can be said that the biosensor is sensitive, accurate, cheap and time saving method assessing nitrate levels in just 30 seconds and can be used as an alternative method for routine analysis of nitrate in environmental samples.

The field of biosensors has endless application and hence it is an emerging field where research and development should take place for cost effective and fast analysis of various components.

This research is just the beginning for development of biosensors and it will help in globalization of the methods available for testing of various components which may be expensive, time consuming and laborious.

## REFERENCES

1. Construction of Nitrate Reductase biosensor using Lab - on - Paper (L-O-P) Technology, Masters diss., Mumbai University, Mumbai,2013.
2. Albanese D, et.al. Screen printed biosensors for detection of nitrates in drinking water, ESCAPE20 2010.
3. Almeida M, et.al.Nitrite biosensing via selective Enzymes – a long but promising route, Sensors 2010; 10: 11530 – 11555.
4. Boncheva M, et al. Self-assembly and applications of biomimetic and bioactive peptide - amphiphiles, Langmuir 206; 12: 5636.
5. Graziana B, et al. Deoxyribonucleic acid (DNA) biosensors for environmental risk assessment and drug studies, Analytica Chimica Acta 2006; 573: 81 – 89.
6. Laura Gonzalez M, et al. Advanced printing and deposition methodologies for the fabrication of biosensors and biodevices, Analyst 2010; 135 :845-67.

7. Alberada Miquel S, et. al. Pesticide determination in tap water and juice samples using disposable amperometric biosensors made using thick film technology, *Analytica Chimica* 2001; 442: 35 – 44.
8. Masatoshi H, et. al. Dependence of a response formed in a micro flow channel on structural and conditional parameters, *Biosensors and bioelectronics* 2005; 21: 2224 - 2231.
9. Chen J.C et. al. Disposable single use electrochemical sensors for the detection of uric acid in human whole blood, *Elsevier, Sensors and Actuators*, 2005; 110: 364 - 369.
10. Berberich J.A, et. al. A stable three enzyme creatinine biosensor and the analysis of the impact of silver ions on creatine amidinohydrolase, *Science Direct, Acta Biomaterialia*, 2005; 1: 183 - 191.
11. Rowe C, et. al. Array biosensor for simultaneous identification of bacterial, viral and protein analytes, *Anal.Chem* 1999; 71: 3846 - 3852.
12. Sapsford Kim E, et. al. Biosensor Detection of Botulinum Toxoid A and Staphylococcal Enterotoxin B in Food, *journal of applied and environmental microbiology* 2005; 5590 - 5592.