Bulletin of Electrochemistry 16 (1) January 2000, pp 25-28

0256-1654/2000/$ 3-50

ACETOBACTER PEROXYDANS BASED ELECTROCHEMICAL BIOSENSOR FOR HYDROGEN PEROXIDE.

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Hydrogen peroxide (H_2O_2) has a great ability to oxidize, bleach and sterilize. It is being used in diary industry, biotechnological processes, wastewater treatments etc. Hence the determination of H_2O_2 is of great importance in these fields. This paper describes microbial based bioelectrode, obtained by coupling immobilized living Acetobacter peroxydans (bacterial cells) to the amperometric oxygen electrode, for the determination of H_2O_2. The fabricated biosensor utilizes the amperometric sensing of oxygen produced during the catalytic decomposition of H_2O_2 at the sensing electrode membrane interface. This sensor has a linear range of detection from 0.1 to 9.5 m*M* H_2O_2 with a response time (T 90) of 60s. The sensor performs well at ambient temperature and in the *p*H range of 5.0 to 8.0. The life period of the sensor is over 90 days. The assimilation studies with most of the organics such as sugars, aminoacids, alcohols, aldehydes etc expected to have no influence over the performance of the probe. The concentration of H_2O_2 upto 11m*M* is found to have no toxic effect on the sensor performances.

Key words: Biosensor, hydrogen peroxide sensor, and microbial sensor

INTRODUCTION

Hydrogen peroxide (H_2O_2) has a great ability to oxidize, bleach and sterlize. It is being used in diary industry, bio technological process, wastewater treatment etc. Hence the determination of hydrogen peroxide is of great importance in these fields. Direct estimation of H_2O_2 by existing electro-analytical methods, which make them less selective to H_2O_2 and prone to interferents present in the sample. The selectivity and also sensitivity can remarkably be enhanced by exploiting the concept of biosensing, where in a biocatalyst is employed in combination with transducer.

Sensors using catalase / peroxidase as the bio catalyst were developed [1-6] based on direct electron transfer at the electrochemical interface to the enzyme [7] and through a redox mediator have been reported. These sensors are selective toH_2O_2. However, the lifetime of this sensor is short owing to the enzyme in stability [8]. Further the enzyme-based sensor requires elaborate procedures for immobilization and fabrication.

In order to have a H_2O_2 sensor with prolonged life, attempts have been made to use animal or plant tissue or whole cells, namely, bovine liver tissue [9] human erythrocytes [10], kohlrabi [11], grape tissue [12], pineapple tissue [13] as biocatalysts which are known for their high catalase activity. These sensors have extended lifetime as anticipated however the sensitivity of the sensor is lost due to the presence of other enzymes. In order to have a suitable microbial strain having high selectivity, sensitivity, stability etc, the results obtained with Acetobacter peroxydans are presented and discussed here.

EXPERIMENTAL

Culture, isolation and immobilization of microorganism

The microorganism Acetobacter peroxydans (NCIM 2144) was procured from National collection of industrial microorganism, National Chemical Laboratory, Pune (India) and subcultured in the nutrient bath containing 5 % sorbitol and 0.5% yeast extract at 298 K. Then the microorganisms were harvested by centrifuging the culture. The Microbial cells were then washed with sterile water followed by phosphate buffer (*p*H 7.0).

Cellulose nitrate membrane (pore size 0.25 μ. Millipore. USA) was used as the matrix for immobilization. The microbe (= cell loading 1 mg dry wt cm - 2) was immobilized by physisorption. The microbe immobilized membrane was sandwiched between dialysis membrane and gas permeable membrane of DO probe, using 'O' ring. The sensor was stored in 0.1*M* phosphate buffer (*p*H 7.0) at 277 K when not in use. The dissolved oxygen (DO) probe used in this study was a modified three-electrode version and was reported earlier [14].

Evaluation of H_2O_2 biosensor

H_2O_2 (30 v/v% volume solution, GR grade, was obtained from BDH, India) was estimated by iodometry from which samples of known concentrations were prepared on a day to day basis. A Wenking Potentiostat (Model PO 573) was

used with a Rikadenki X - y - t recorder for the amperometric measurements.

5 ml of phosphate buffer (*p*H 7) was added to the light protected, thermostatically controlled reaction cell under constant stirred condition. The sensor was inserted into the solution. The oxygen steady state reduction current was monitored at - 0.6 V against Ag/AgCl reference electrode. After oxygen reduction current reaches a steady state, required H_2O_2 samples from the stock were added to the cells stepwise manner and the current was monitored as a function of time.

To study the influence of *p*H on the sensor performances 5 ml of respective buffer solution, phthalate buffer of *p*H 4 and 5, or phosphate buffer of *p*H 6 to 8 or borax buffer of *p*H 9, was taken in the reaction cell then the sensor was placed in the buffer for 15 mts before the injection of H_2O_2. To study the effect of temperature the temperature of the reacting cell was brought as required using thermostated water bath. The sensor was placed in the cell for 15 mts before the addition of H_2O_2.

RESULTS AND DISCUSSIONS

Electrode response to H_2O_2

Fig.1 shows the response of the biosensor for the stepwise addition of 200 (l of 5 m*M* H_2O_2. The initial steady state base current (I_{so}) is attributed to the endogenous respiratory activity of the immobilized microbial cells. i.e. when the oxygen present in the bulk solution is passing through the microbe immobilized membrane, the microorganism utilizes only a fraction of D.O.. When H_2O_2 is added, the microbe decomposes the H_2O_2 and thereby liberating more oxygen at the electrode vicinity. The above catalytic action can be envisaged as arising due to the action of constituent enzymes (such as catalase and peroxidase) which protect the microorganisms by decomposing the toxic peroxides and super oxides [15-17]

$$2H_2O_2 \xrightarrow[\text{catalyse}]{\text{microbial}} H_2O + O_2 \qquad (1)$$

When the rate of decomposition of H_2O_2 by bacterial cells attain equilibrium, the current output also attain a new steady state (I_{s1}).

It is relevant to mention that microbial sensor of respiratory type electrodes take longer response time (300 s) and is attributed to the intracellular activity, namely, assimilation of the organic substrates. The response time of the present H_2O_2 sensor is much faster (100 s) indicating that the enzymatic decomposition of H_2O_2 is essentially taking place at the cellular walls as the microbes are capable of decomposing both intracellular and intercellular H_2O_2 [18]. From the calibration graph of Fig 2 it is evident that A.peroxydans based sensor can detect 0.2 m*M* of H_2O_2 with a perfect linearity upto 11 m*M* concentration of H_2O_2. This response of H_2O_2 biosensor is in the range of 0.02 m*M* to 11.0 m*M* which can be described by the equation [19]

$$Y = 41.5\ K \qquad (2)$$

Where Y is the oxygen pressure in Kpa and K the H_2O_2 concentration in m*M* l^{-1}. The detection limit of 10^{-4} *M* l^{-1}. is considered to be sufficient for practical use (19,20). The

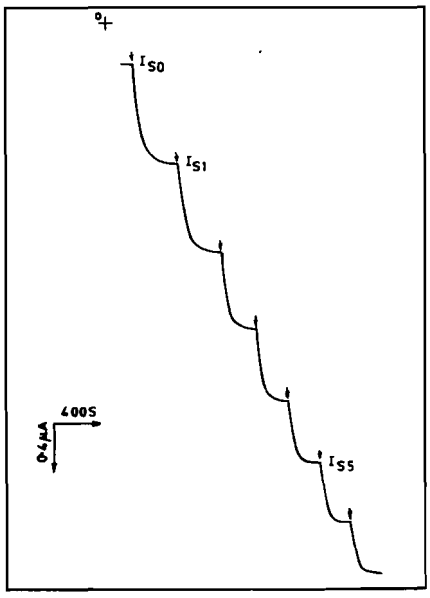

Fig.1: Typical response curve of A.Peroxydans baased sensor for the subsequent additions of 200 μl of 50mM H_2O_2 to the cell containing 5 ml of phosphate buffer (pH 7.0)at 298K.

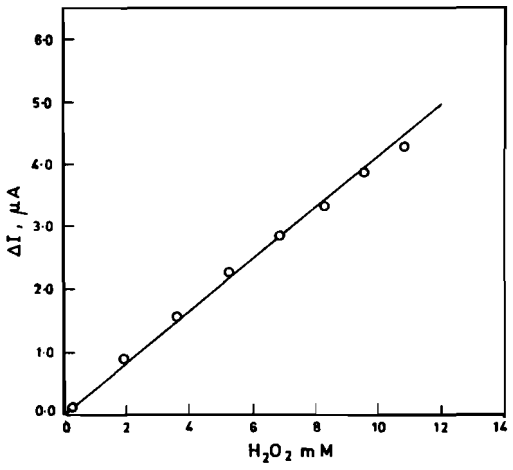

Fig.2:Calibration plot of ΔI Vs H_2O_2 concentration

TABLE I: Amperometric response of the *A.Peroxydans* based sensor to the various organics.

Organic substrate 20ppm	Δ I* (nA)	Comp. to 20 ppm of glucose in %
D-glucose	42.9	100
Sucrose	52.8	123
Soluble starch	52.8	123
Lactose	26.4	62
fructose	52.8	123
Glutamic acid	34.3	80
L-Alanine	30.8	72
Glycine	6.8	16
Methanol	0	0
I-Propanol	82.3	192
Citric Acid	0	0
Lactic Acid	75.9	177
Acetic Acid	0	0
Urea	13.7	32

** Decrement in the cathodic current arises due to the respiratory activity*

present detection limit is due to the use of oxygen saturated solutions; this can be improved using oxygen free solutions.

Microbial loading

Fig 3(a) shows response characteristic of H_2O_2 sensors of having different microbial cell loading when stored in buffer *p*H 7.0 at 277 K when not in use. Response to H_2O_2 is increasing with cell loading. when the cell loading is > 1 mg cm^{-2} (dry weight) the electrodes show a maximum current output. Instability in the current output observed when electrodes posses cell loading of 1 mg cm - 2. At the cell loading of 2.0 mg cm^{-2} sensor starts to give stable response early.

When the microbial electrodes were stored in phosphate buffer containing 1 m*M* H_2O_2, the current out put as well as the time taken to reach a new steady state. I_{s1} is almost same in all the electrodes irrespective of cell loading. This kind of biosensor performance can be attributed to the enhancement of the microbial growth and or enzyme activity with time [21] under this condition.

Long term stability

Fig 3(b) shows the long term performance of the H_2O_2 biosensor. When compared with unmediated enzyme catalase based sensor, whose response was stable only for 8 days [19,22], the present biosensors have life time over 18 months, because of retaining enzyme activity in the living cell it self. The long term stability of the sensor in buffer *p*H 7.0 at 277 K has good agreement with the finding with lettuce seed meal [23] for the estimation of H_2O_2.

Tolerance to H_2O_2

Fig 3(c) shows the typical response of the H_2O_2 sensor in 11 m*M* H_2O_2 solution for 2 hrs. This proves that even long exposure at 10 m*M* H_2O_2 will not denature the microbial activity.

Effect of *p*H

Fig 4 explains the performance of the H_2O_2 biosensor in the *p*H range of 4 to 9. The sensor functions well in the mid *p*H range of 5 to 8. which is better than the reported value [24] Exposure of the sensor in the *p*Hs of below 5.0 and above 8.0 is found to denature the microbial activity.

Influence of temperature

Fig 4 also shows the performance of the bio-electrode in the temperature range of 283 to 313 K with the raise in temperature a gradual increase in the current out put of the sensor has been observed, which could be due to the

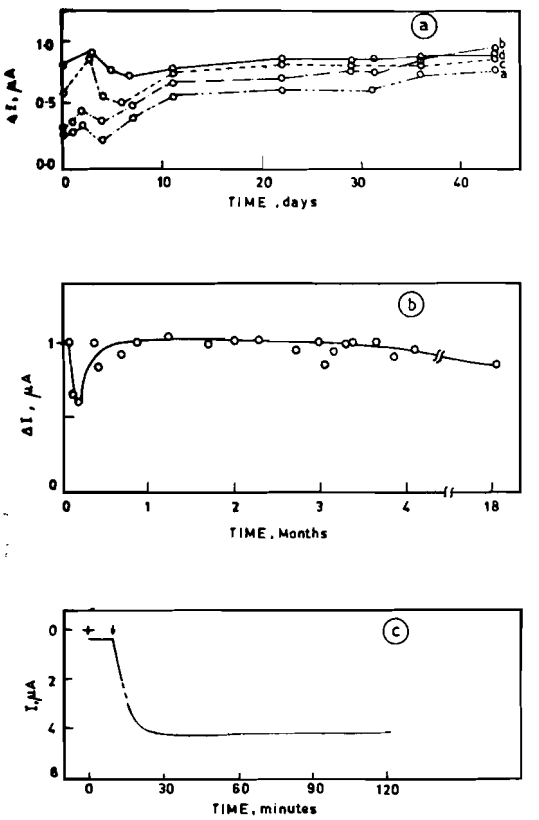

Fig.3a. Long term response of A.Peroxydans based sensor for 2 mM H_2O_2, in 0.1M phosphate buffer pH 7.0 at 298 K when having cell loading (dry weight) of (a) 0.25, (b) 0.50, (c) 1.0 and (d) 2.0 mg / cm^{-2}.
Fig.3b. Long term stability of the H_2O_2 sensors (cell loading 1mg /cm^{-2}, dry weight, at 298 K).
Fig.3c. Response of A. Peroxydans based biosensor for 11 mM of H_2O_2.

combined activity of auto catalytic decomposition of H_2O_2 with temperature and decomposition by microbial biocatalyst.

Assimilation studies

Table I shows the assimilation capacity of A. peroxydans for a wide spectrum of organics under aerobic condition along with the comparision for D - glucose in order to generalise the results. The presence of other organics in the analite causes an increase in the respiratory activity of the microbe resulting a decrease of D. O. concentration at the electrode vicinity. This decrease in the current out put is the function of substrate (organics) concentration. The results of assimilation test for various organics show a good correlation with several studies on growth and other physiological studies reported earlier. [25-30], whereas the current response for H_2O_2 is increased with concentration. For 20 ppm of H_2O_2 response is + 300 nA, but for different organics it is (- 40nA) decreasing. Hence the assimilatory activity of these microbe will not have much influence on the performance of H_2O_2 sensor.

CONCLUSIONS

A peroxydans can be effectively used in the biosensor for H_2O_2. The sensing range and working conditions of the bioprobe is enough for most of the practical applications. Further this microbe is stable even at concentration level of 11 m*M* of H_2O_2. Organics present in the analyte is also expected to have no significant effect on the performance of the sensor. Sensitivity of the probe can be improved by using deaerated buffer solution and culturing the microbe in the medium containing H_2O_2. This bioprobe method can also be used as an easy tool to assess the availability/activity of the enzyme catalase from the microbial cells.

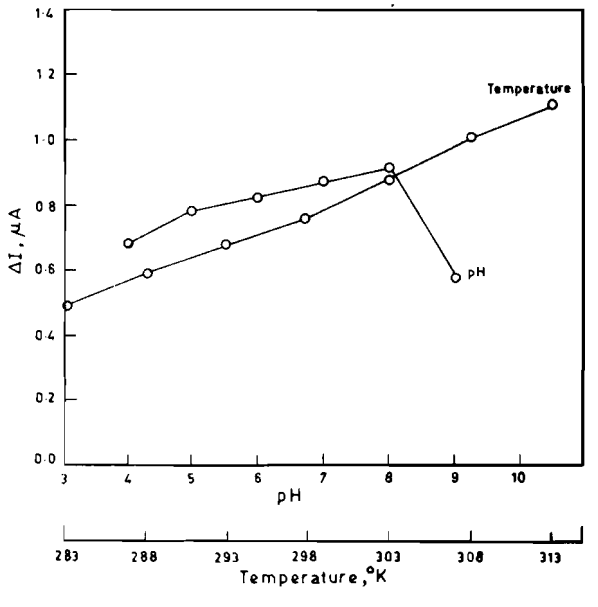

Fig.4:Effect of pH and temperature variation on the performance of A. Peroxydans based sensor for 2 mM H_2O_2.

Acknowledgement: One of the authors Mrs. S.R thanks CSIR for the financial support.

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