DEVELOPMENT OF ENZYMATIC BIOSENSOR FOR THE DETECTION OF AMMONIUM

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ABSTRACT

Monitoring of ammonium is essential since excess presence of it either in the air or aquatic environment or food samples is toxic. Use of biosensors for monitoring ammonium is advantageous due to their high selectivity and sensitivity. In the present study the enzyme alanine dehydrogenase was used for the development of ammonia biosensor since this enzyme is more stable and responds to wide range of ammonium concentrations. This enzyme converts pyruvate to alanine in the presence of ammonium and NADH. During this reaction two electrons will be generated and they will be transferred to the electrode and the signal will be amplified. Alanine dehydrogenase was isolated from Bacillus subtilis and it showed optimum activity at pH 8.5 and 35^{0}C. The enzyme was stable up to 45^{0}C and lost most of its activity at 50^{0}C. By using this enzyme a biosensor was developed by adopting amperometry. Here the working electrode coated with enzyme, reference electrode and counter electrodes were used and the signal generated at the working electrode was measured by using potentiostat. Here, we used screen printed electrode design since it's easy to carry and sensitive to low signal. The biosensor responded to the different concentrations of ammonium suggesting the suitability of this enzyme for the screen printed design of the biosensor.

Keywords : Alanine Dehydrogenase, Amperometry, Biosensor, Pyruvate, Bacillus Subtilis

I. INTRODUCTION

Higher levels of ammonium is hazardous to mammals and aquatic organisms. Elevated levels of ammonium in human blood is indicator of kidney disorder, stomach bacterial infection or liver dysfunction. Consumption of ammonium contaminated food can chronically elevate the concentration of the ammonium ion in the blood which can severely affect the function of brain, cause hyper excitability, coma, convulsions and finally death. Ammonium ions are responsible for aerosol formation and ammonium nitrate is highly used in landmine blasting and explosive preparation. Hence, the sensor which can monitor the ammonium will be helpful in environmental, food, health and defense applications. Metal oxide ammonia sensors are mostly based on SnO_2 and they operate based on the change of conductance due to the chemisorption of gas molecules on the sensing layer. The major drawback with these sensors is they are not selective to the particular gas. Optical sensors depend on the colorimetry or spectrophotometry. Indophenol blue based colorimetric method was used for determining the ammonium concentration. Here ammonium reacts with phenolic agent to form indophenol blue

that absorbs light between 630- 720 nm under oxidizing conditions. Other optical sensors based on Berthelot's reaction [1], silver nanoparticles [2] and Nessler's reagent were developed for sensing ammonia. High-performance liquid chromatography coupled with fluorescence detector was used for the ammonium detection. These conventional methods for the detection of ammonium are time consuming and tedious. In the colorimetric method, phenolic reagent and its by-product of the reaction are highly toxic. Spectrophotometric method will be interfered by other photoactive compounds and chromatographic methods require pre-column derivatization in order to treat the sample for detection. Both optical and chromatographic methods require big instrumentation for the detection of signal.

Potentiometric ammonia sensor based on zirconium titanium phosphate ion exchanger was developed [3]. It requires big instrumentation even though it has long life. An ammonium electrode based on the poly vinyl chloride (PVC) membrane containing palmitic acid and nonactin as an ammonium ionophore for the determination of ammonia was constructed [4]. However this sensor is not specific for the ammonia. Multiwalled carbonnanotube/copper composite paste electrode was developed for the detection of ammonia [5]. But reproducibility was very poor with this electrode. The major problem with above described sensors is they are not very specific to the ammonium and in this context biosensors become important since they depend on the highly specific bioreceptors. Biosensor based on the immobilized nitrifying bacteria (*Nitrosomonas europea*) was used for the detection of ammonia in waste waters. However, lifetime of this sensor is only 2 weeks since living organism itself was used as bioreceptor. Enzyme based biosensors were widely used since their stability can be easily manipulated using stabilizing agents or molecular approaches. Enzyme-based amperometric biosensors gained lot of importance in the last decade due to high selectivity of the bioreceptor and the sensitivity of electrochemical signal transduction. Two enzyme based sensor was constructed for the detection of ammonium by immobilizing glutamate dehydrogenase and glutamate oxidase on a Clark-type oxygen electrode [6]. But this sensor lost around 60% of its response after 18days. Another two enzyme based ammonia sensor was developed based on the glutamate dehydrogenase and diaphorase [7] . But it showed linear response only at lower concentrations of ammonium (2.5-500 μM). Two enzyme based sensors are difficult to manage since each enzyme needs different optimum conditions and it's difficult to maintain both the conditions simultaneously and it affects the stability of the sensor. Hence, single enzyme system i.e. alanine dehydrogenase (AlaDH) was used as a bioreceptor in the present work. This enzyme converts pyruvate to alanine and generates 2 electrons in the presence of ammonia and NADH. The reaction is written below.

$$\text{Pyruvate} + \text{NADH} + NH_4^+ \xLeftrightarrow{\text{AlaDH}} \text{L-alanine} + NAD^+ + H_2O + 2e^-$$

II.MATERIALS AND METHODS

2.1. Microbial growth and conditions

The bacterium, *Bacillus subtilis* (ATCC 6633) was maintained on the nutrient agar (pH 7.0) slants at 4^0C. For the production of AlaDH, the culture was grown at pH 7.0 and 37^0C by using nutrient broth supplemented with L- alanine (0.1 mg/ml) for 24 h.

2.2. Enzyme Assay

The assay mixture (1.2 ml) contained 0.125 mM NADH, 2 mM pyruvate, 100 mM NH_4Cl and 300 µl of appropriately diluted enzyme in the 50 mM Tris-HCl (pH 8.5) buffer. The assay was carried out at room temperature by recording the decrease in absorbance of NADH at 340 nm. Enzyme activity units were measured as µmol of NADH hydrolyzed min^{-1}.

2.3. Stabilization studies

Different stabilizers like glycerol, EDTA, poly vinyl alcohol, poly vinyl acetate, poly vinyl pyrrolidone etc.were used for studying their effects on the enzyme activity at the storage temperature of 37^0C for different periods of time.

2.4. Enzyme Purification

Bacterial cells were lysed by sonification and the cell lysate was subjected to 80% ammonium sulfate precipitation and the precipitated protein was ran on DEAE-cellulose and Sephadex-G-100 chromatographic columns. The final sample was ran on 10% SDS-polyacrylamide gel to check the purity of the enzyme.

2.5. Biosensor construction and response studies

This biosensor was constructed based on amperometry. A mixture of polyHEMA, NADH, sodium pyruvate, EDTA and alanine dehydrogenase enzyme was prepared. The mixture was then deposited onto the carbon working electrode of screen printed electrode system and allowed to dry at 4^0C overnight. Carbon counter electrode and silver reference electrode were used in the system. The electrolyte (50 mM Tris-HCl, pH 8.5) containing different concentration of ammonium was placed on the electrode system such a way that it covers all 3 electrodes. Then the electric signal was measured with the help of potentiostat. Empty electrolyte was used as a control.

III. RESULTS AND DISCUSSION

This ammonia biosensor depends on the activity of AlaDH and this enzyme generates two electrons in the presence of ammonium and NADH while converting pyruvate to alanine. Generated electrons were detected by adopting amperometry since it's very sensitive technique and three electrodes were used during this process. They were working electrode, counter electrode and reference electrode. These electrodes were screen printed and coating of AlaDH along with other reagents on the working electrode will enhance the transfer of generated electrons to the working electrode and this will lead to the sensitive detection of electrons generated in response to the ammonia. The pure AlaDH is required for the construction of this ammonia biosensor in order to get high selectivity to the ammonium. Hence this enzyme was purified from *B.subtilis* and characterized.

For the production of AlaDH, a loopful of *B.subtilis* culture was inoculated in to the 100 ml nutrient broth (NB) containing L-alanine (0.1 mg/ml) and grown at 37^0C (pH 7.0, 150 rpm) for 15h. Four ml of inoculum was added to the 200 ml of NB broth with alanine and grown at the same conditions for 17h. Completely grown

culture was pelleted by centrifuging at 5000 rpm for 20 min. Pellet was washed twice with 100 mM Tris-HCl buffer (pH 8.5) and resuspended in the same buffer containing PMSF and EDTA. Then cells were lysed by sonification (250 W, 30 KHz) and cell debris was removed by centrifuging at 10000 rpm. The supernatant was checked for the AlaDH activity by following the standard enzyme assay. For the purification of AlaDH, the supernatant was precipitated with 80% of ammonium sulphate and the precipitate was dissolved and dialysed against the Tris-HCl buffer (100 mM,pH 8.5) at 4^0C. The dialysed sample was loaded on to the DEAE-cellulose ion exchange column and eluted with 0.2-1.0 M NaCl. The fractions showing the major enzyme activity were pooled together and dialysed against the buffer and ran on the Sephadex-G-100 column and the fractions showing major AlaDH activity were pooled and lyophilised. The purity of the enzyme was checked on the 10% SDS-polyacrylamide gel (Fig.1).

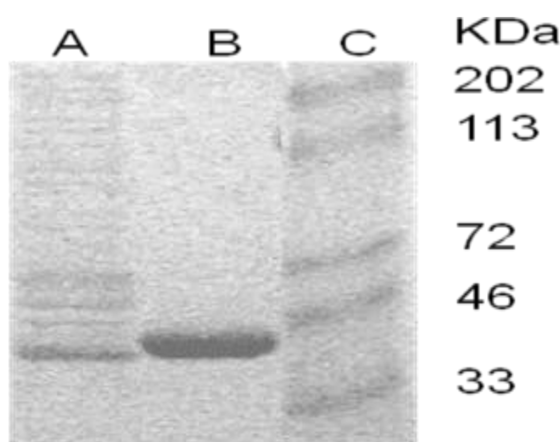

Fig.1. 10% SDS-polyacrylamide gel showing the purified protein (lane B). Lane A: crude extract,

The presence of single band (Fig.1, lane B) in the purified sample suggest the homogeneity of the AlaDH and this enzyme moves at the molecular mass of 42 KDa on the 10% SDS-polyacrylamide gel. The purified AlaDH showed the optimum pH of 8.5 (Fig.2) and the optimum temperature of 35^0C (Fig.3) when the effect of different pH and temperatures on the activity of the enzyme was studied. Enzyme lost its activity significantly at acidic pH and its activity started decreasing after pH 8.5 (Fig.2). Enzyme showed the increase in activity up to 35^0C and it remains more or less constant up to 45^0C and it showed drastic decrease in activity afterwards (Fig.3). Optimum enzyme activity around ambient temperatures is advantageous for the sensor since ammonium measurements will be done at ambient temperatures in real time.

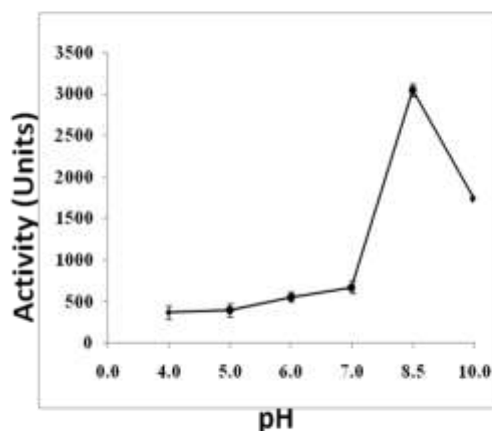

Fig.2. Graph showing the effect of pH on the activity of alanine dehydrogenase

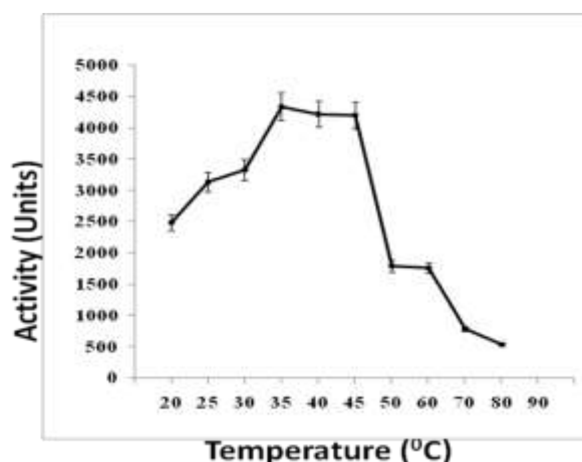

Fig.3. Graph showing the effect of temperature on the activity of alanine dehydrogenase

Studies on the storage stability of AlaDH for different periods around ambient temperature are required since this biosensor depends on the activity of AlaDH. When these studies were conducted in the presence of different stabilizers at 37^0C, the enzyme could retain the 97.9% and 76.44% of original activity in the presence of glycerol and EDTA respectively on the 30th day of storage (Table 1). However, in the absence of stabilizer the enzyme could retain only 68.08% activity on the 30th day and other stabilizers (poly vinyl alcohol, poly vinyl acetate and poly vinyl pyrrolidone) could also retain the more or less same kind of activity. On the 75th day, the enzyme alone could retain only 6.86% of original activity, whereas in the presence of glycerol and EDTA it could retain 43.97% and 46.76% activity respectively (Table 1). These results suggest that glycerol and EDTA acted as good stabilizers for AlaDH. However, calcium chloride, sucrose, poly ethylene glycol, glycine and tween 80 acted as inhibitors for AlaDH (data not shown).

Table 1: Effect of stabilizers on the storage stability of alanine dehydrogenase at 37°C

S. No.	Stabilizer	Residual activity (%) on				
		0 day	15th day	30th day	45th day	75th day
1	Control	100	70.44	68.08	63.45	6.86
2	EDTA	100	87.72	76.44	65	46.76
3	Glycerol	100	85.14	97.9	60.11	43.97
4	Poly vinyl alcohol	100	65.75	62.28	58.99	3.33
5	Poly vinyl acetate	100	63.49	60.11	49.72	5.80
6	Poly vinyl pyrrolidone	100	64.22	62.15	60.66	7.43
7	Poly vinyl alcohol + Poly vinyl pyrrolidone	100	71.41	69.75	67.71	7.12
8	Poly vinyl alcohol+ Poly vinyl acetate	100	67.85	77.92	66.41	9.45

Ammonia biosensor was constructed by coating a layer of solution containing AlaDH, polyHEMA, NADH, sodium pyruvate and the stabilizer EDTA on the working electrode of the screen printed electrode system. After drying, the electrolyte containing different concentrations (0-100 mM) of ammonium was placed on the working electrode and the electric signal generated at the working electrode was detected by plotting cyclic voltagram with the help of potentiostat. When there is no ammonium, the cyclic voltagram was at basal level (Fig.4, CV 1) and it didn't show any signal, whereas at 5 mM ammonium the cyclic voltagram was above the basal level (Fig.4, CV 2) and the signal proportionately increased with the increasing concentrations of ammonium (Fig 4; CV 3, 4, 5 and 6 correspond to 10, 25, 50 and 100 mM ammonium respectively) suggesting the biosensor response to the different ammonium concentrations.

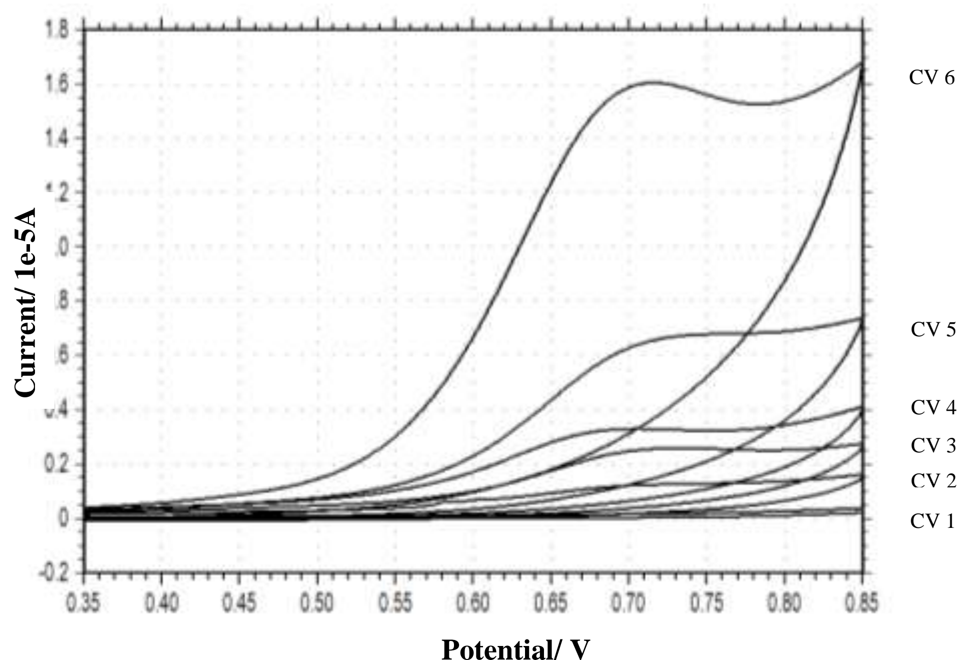

Fig.4. Cyclic voltagrams of biosensor in response to the different concentrations of ammonium. CV 1, 2, 3, 4, 5 and 6 are the cyclic voltagrams corresponding to the 0, 5, 10, 25, 50 and 100 mM ammonium.

IV.CONCLUSIONS

The developed biosensor for the detection of ammonia works at ambient conditions. Improvising the biosensor for working at extreme conditions could be advantageous, even though ammonia levels were commonly checked at ambient conditions. The electric signal obtained while detecting the ammonium was in terms of 10^{-5} amps and this signal should be improved in order to calibrate the biosensor for wide range of ammonium concentrations. Nevertheless, this sensor clearly differentiated the ammonium concentrations in the range of 0-100 mM. Stability of this sensor after different storage periods need to be checked even though this study was carried out for the bioreceptor, AlaDH.

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