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Simple, qualitative cum quantitative, user friendly biosensor for analysis of Urea

Neelam Verma* Rajiv Kumar Minhas Sachin Kumar

Department of Biotechnology, Punjabi University, Patiala, Punjab, India

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ABSTRACT

Present study reports the development of simple, user friendly qualitative cum quantitative biosensor based on urease enzyme for the analysis of urea. Enzyme was isolated from a urease producing microbe originally isolated from National Fertilizer Ltd., Bathinda, which was later found to be a Corynebacterium spp. (MTCC 8143), having log phase between 9-24 hrs. Highest enzyme activity was found to be at 21 hrs. To fabricate biosensor crude enzyme extract and indicator (phenol red) was co-immobilized on nylon membranes with TEOS based hydrosol-gel. Membranes with immobilized biocomponent and indicator when dipped in urea solution, color changed from yellow to red-violet. Time taken for the change in color (response time) was correlated with urea concentration. Urea could be detected in the solution with a detection limit as low as 10^{-9}M with a response time 2 min 29 seconds. Constructed biosensor was applied to human blood and urine samples. Bio-component was stable for 20 days when kept at 4^0C.

Keywords: Urease, *Corynebacterium spp.* (MTCC 8143), Urea Biosensor, Hydrosol-gel, Phenol Red, Allyl alcohol, 2-propanol.

INTRODUCTION

Being a waste product of many living organism and the major organic matter of human urine, analysis of urea has got its clinical importance. A build up of urea in blood is known to be fatal. Therefore urea is important to be checked at both the levels *i.e.* in the food stuff as well as in clinical samples *e.g.* blood and urine [1-4].

Biosensor is an analytical device that integrate a biological component *i.e.* enzyme, antibody, DNA or the whole cell etc. with a transducer to produce a measurable signal corresponding to the analyte concentration. Enzymes being specific, easy to isolate and relatively cost effective, are widely used bio-component to construct a biosensor. In aqueous solutions enzymes lose their catalytic activity rather rapidly because enzyme can suffer oxidation reaction or its tertiary structure could be destroyed at air/water interface [5]. These problems can be minimized considerably by enzyme immobilization to an inert support material; bioactive molecule may be rendered insoluble, retaining its catalytic activity thereby extending their useful life [6-8].

In view of this necessity and advantage, since 1960s a variety of techniques have been developed to immobilize biological molecules including adsorption, covalent attachment and entrapment in various polymers. In general adsorption techniques are easy to perform but the bonding of bio-molecules is often weak and such biocatalysts lack the degree of stabilization and easy leakage from the matrix. Perhaps it is possible with entrapment or covalent binding methods, but the covalent linkage method is tedious, often requires several chemical steps and sometime the compounds involved inactivate or reduce the activity of biomolecule. Therefore direct immobilization of active biological component e.g. enzyme, protein, cells and antibodies etc., in porous metal oxide carrier by physical

entrapment via sol-gel process has drawn great interest in recent years [9-14]. The two major advantages of with a sol-gel system is that it can retain large content of water which make the encapsulated bio-component stable for long term [15] and the process can be performed at room temperature [16]. In the present study, urease enzyme was used as a bio-component, isolated from *Corynebacterium spp.* (MTCC 8143). The enzyme was immobilized with sol-gel approach onto a nylon membrane to construct the biosensor; constructed biosensor was applied to urine and blood samples.

MATERIALS AND METHODS

Reagents & Culture

All chemicals used were of analytical grade. The culture used in the study is an isolate, isolated in Biosensor Technology Lab, (Dr. Neelam Verma) Department of Biotechnology, Punjabi University, Patiala, identified by IMTECH, Chandigarh to be *Corynebacterium spp.* (MTCC 8143). Culture was grown for isolation of enzyme in medium composed of Beef extract (10g/L), Peptone (10g/L), NaCl (5.0g/L) and Urea (25g/L) with a pH 7.0-7.5 at 37^0C, maintained by further sub-culturing after 30 days and stored at 4°C. Growth profile of culture was studied by taking OD at 600nm after every 3 hour up to 36 hrs, enzyme activity was assayed with Nesseler's method.

Enzyme Extraction & Assay

200ml of 21 hr old culture was centrifuged at 5000 rpm for 20 minutes at 4^0C, supernatant discarded, pellet was washed with 2 ml Phosphate Buffer Saline (PBS), centrifuged again and taken in 1 ml PBS. Suspension thus obtained was taken in an eppendorf, sonicated (pulse rate 25sec-on 10sec-off with 50% amplitude) and centrifuged at 5000 rpm for 5 minutes to get the supernatant. Supernatant is considered as crude enzyme extract and used for further study. Enzyme activity was assessed with Nessler's reagent (reaction represented as follows).

$$2\ K_2[HgI_4] + NH_3 + 3\ KOH \rightarrow I\text{-}Hg\text{-}O\text{-}Hg\text{-}NH_2 + 7KI + \quad 2\ H_2O$$

Immobilization of the Bio-component

For the present study, urease was immobilized by TEOS-Hydrosol-gel method [17].

Preparation of a TEOS sol-gel

Sol-gel was prepared by mixing 60 µl of distilled water, 600 µl of alcohol, 50 µl of TEOS, 10 µl of 5mM NaOH, and in a small test tube at room temperature. This sol-gel solution was cooled and kept at 4^0C immediately after mixing.

Co-immobilization of enzyme and phenol red onto nylon membranes

Nylon membranes were cut into suitable sizes (2 X 2 cm). 100 µl of 2% phenol red and 50 µl of enzyme was added to the chilled TEOS sol-gel and mixed well. This mixture was put onto nylon membranes and membranes were allowed to dry for one hour.

Preparation of Standard Curve and Effect of alcohol chain length

Membranes with immobilized enzyme and indicator (phenol red) were dipped into urea solutions of known concentrations ranging from 2 M to 10^{-9} M and time taken for color change was noted down. Effect of increasing chain length of alcohol used in hydrosol-gel preparation on response time was also studied. Different alcohols used in the study include ethanol, 2-propanol and allyl alcohol.

Application & Storage stability of Biosensor

Urea, an end product of nitrogen metabolism has great significance in clinical chemistry where blood urea nitrogen is an important indicator of possible kidney malfunction. Apart from this urea also destabilizes biological macromolecules, altering their structure and function. Urea biosensor finds extensive applications in clinical analysis [18] therefore biosensor developed in present study was applied to urine and blood samples from normal healthy people. Blood serum was used for the analysis, before the analysis pH of both, urine and blood samples was set to 6. Storage stability of the nylon membranes with immobilized enzyme, change in color of nylon membranes and enzyme activity was checked at 4°C for several days to determine their storage stability.

Validation of developed biosensor

Prepared membranes was tied at the tip of ammonium ISE electrode, was dipped into urea solutions of different concentration from 2 M to 10^{-9} M. Potential was noted down after incubation of 2 minutes.

RESULTS

In the growth profile lag phase was ended within 3-9 hrs, after that log phase starts which last up to 21 hrs (Fig. 1). Enzyme activity was maximum i.e. 0.183 unit/ml at 21 hrs.

Effect of alcohol used in hydrosol-gel on Response Time

There is a significant decrease in response time with increase alcohol chain length though the effect shrinks with lower urea concentrations. (Table-1)

Color of membranes having immobilized enzyme with sol-gel prepared from different alcohols (2-propanol and Allyl alcohol) changes with different concentrations of urea is shown in Fig-3.

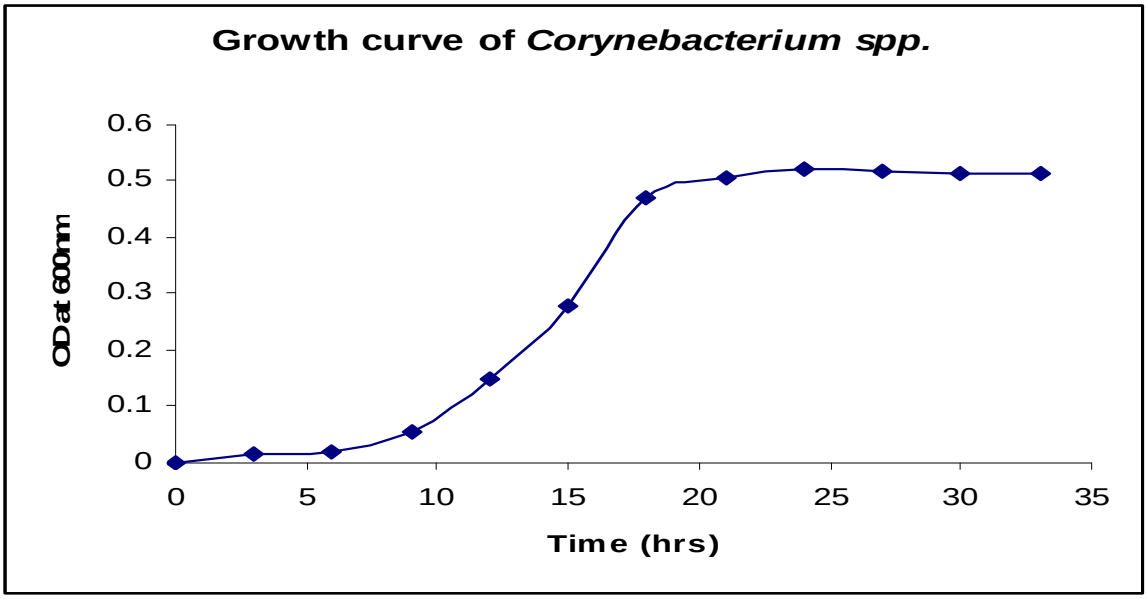


Fig 1:- Growth profile of *Corynebacterium sp.* (MTCC 8143).

Table: 1. Response time decreased with increase in chain length of alcohol used in hydrosol-gel preparation for all the concentrations of urea (2M – 10^{-9}M)

Urea solution concentration (M)	Response time in Seconds (Ethanol)	Response time in Seconds (2-Propanol)	Response time in Seconds (Allyl Alcohol)
2	50	41	31
1	54	47	36
10^{-1}	70	61	46
10^{-2}	80	70	59
10^{-3}	86	78	69
10^{-4}	96	92	84
10^{-5}	106	104	98
10^{-6}	124	117	115
10^{-7}	134	128	124
10^{-8}	140	141	135
10^{-9}	152	149	149

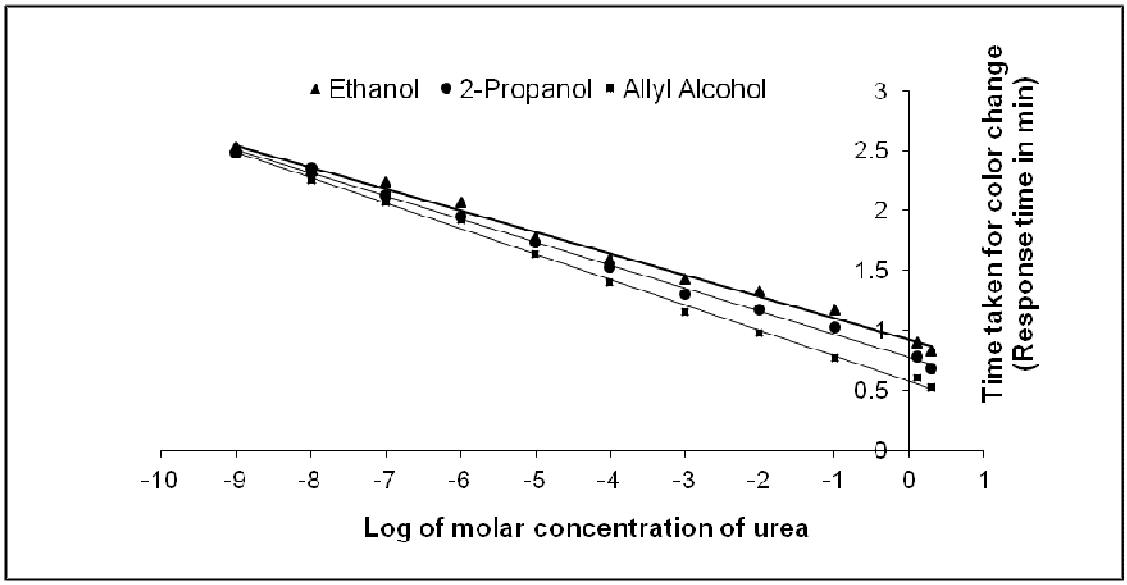
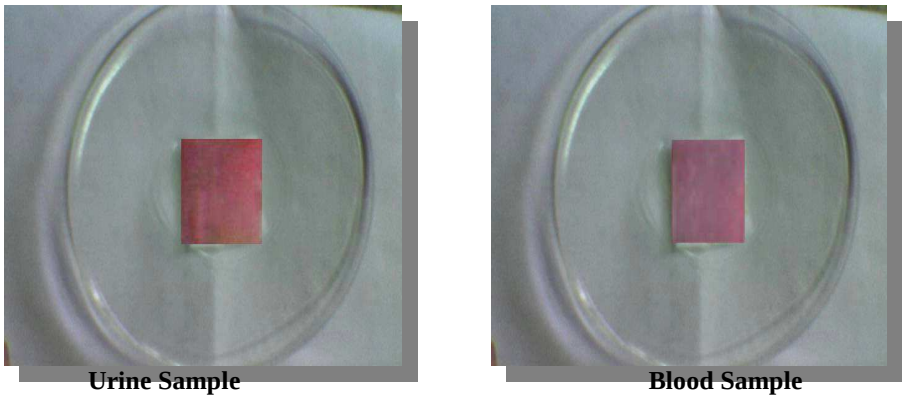


Fig 2:- Graphical representation of performance of biosensor with urea standards (2M – 10^{-9}M), a linear relation is shown between time taken for change in color and concentration of urea. Response time has been decreased with increase in chain length of alcohol used for sol-gel preparation.

Fig.3:- Color of membranes prepared with different sol-gel 2-Propanol and Allyl Alcohol after reaction (Urea Solution 2M -1O^{-9} M)

Table: 2. Normal Blood and Urine Samples

Sample number	Response time in Seconds (Blood)	Estimated Urea Conc.	Response time in seconds (Urine)	Estimated Urea Conc.
Sample 1	74	$7.8x10^{-4}$M	39	0.44M
Sample 2	65	$4.0x10^{-3}$M	41	0.31M
Sample 3	67	$2.8x10^{-3}$M	38	0.52M
Sample 4	69	$1.9x10^{-3}$M	43	0.21M
Sample 5	71	$1.3x10^{-3}$M	37	0.63M
Sample 6	67	$2.8x10^{-3}$M	44	0.17M



Urine Sample **Blood Sample**

Fig 4:- Color of membranes in normal urine and blood samples

Table 3: Increase in potential with increasing Urea Concentration

Urea concentration(M)	**mV reading after the reaction**
2	-83.3
1	-96.1
10^{-1}	-114.2
10^{-2}	-124.5
10^{-3}	-136.5
10^{-4}	-145.4
10^{-5}	-153.2
10^{-6}	-160.2
10^{-7}	-172.5
10^{-8}	-185.5
10^{-9}	-193.2

Estimation of Urea level in Blood and Urine samples

Response time varied between 37-74 seconds for all samples (Table-2), urea concentrations estimated with response time from standard curve (Fig-2) appear in normal range. Color change of membranes with blood & urine samples is shown in Fig-4. Normal range of urea in blood is $2.5x10^{-3}$ M to $6.6x10^{-3}$M while in urine is 0.25M to 0.6M [19]

The nylon membranes having immobilized enzyme were kept at 4°C for several days and it is found that, the nylon membranes remain in unchanged condition and bio-component is active for 20 days.

Validation of Biosensor performance

Hydrosol-gel membranes were prepared in propanol and concentration of ammonia released was determined quantitatively using ion selective electrode. A hydrolysis time of 2 minutes was given and mV reading was noted down after the reaction (Table-3). There is an increase in potential with increase in urea concentration as increase in ammonium ion produced.

DISCUSSION

Miniaturization and Portability

In general, there is a growing tendency toward miniaturization of analytical systems, because it allows the handling of low-volume samples, a reduction in reagent consumption and waste generation moreover increases sample throughput [20]. Taking advantage of miniaturization benefits, sensors and biosensors can become inexpensive and easy-to-handle analytical devices for fast, reliable measurements of chemical species. Biosensor miniaturization has a particular significance for clinical applications when sometimes an implantable sensor is desired for continuous in vivo monitoring [21] for pharmaceutical industries, in the field of high-throughput screening [22]. Membranes developed in the present study are stable for 20 days; portability is not a problem at all.

Cost Effectiveness

Jie *et al*., [23] developed macropore-sized (100 µm) sol-gel bio-glasses using poly vinyl alcohol (PVA) as a pore-forming agent. Soares *et.al*, [24] entrapped *Candida rugosa* lipase in TEOS and MTMS (methyltrimethoxysilane) in presence of PEG. Keeping in mind high cost involved in reagents used in above discussed studies present work involved the use of alcohols instead of PVA and PEG in order to make the process cost effective.

Response Time

Biosensor developed by Jha *et al*., [25] based on urease immobilized in PVA-PAA composite matrix for estimation of blood urea nitrogen (BUN). The sensor working range was 1-1000 mM urea with a response time of 120 seconds. Present biosensor has got a quicker response.

Recently urease based biosensor fro urea analysis developed by Dindar *et.al,* [26]. Urease enzyme used in the study was of *Helicobacter pylori*, isolated from biopsy sample obtained from antrum big curvature cell extracts. Linear working range reported is 10^{-2} to 10^{-5}M with a response time 1-2 minutes.

An amperometric urea biosensor developed by Bozgeyik *et.al,*[27] immobilizing urease onto poly (N-glycidylpyrrole-co-pyrrole) conducting film by direct covalent attachment, reported to respond in 4 seconds only with a linear range of 0.1 to 0.7 mM.

CONCLUSION

In nutshell present work resulted in the development of a rapid, portable, miniaturized, disposable, cost effective, easy to use and reliable biosensor with improved response time and a storage stability of more then 20 days at 4^0C. The biosensor has been applied for monitoring urea conc. in blood and urine samples.

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