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PRELIMINARY INVESTIGATIONS ON AN ENZYME IMMOBILIZED OPTICAL BIOSENSOR FOR ARSENITE DETECTION

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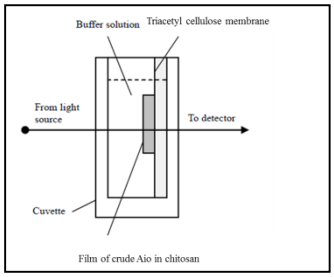
Graphical abstract



Abstract

Arsenite is an inorganic form of arsenic that poses hazardous effect to human. It is a common environmental heavy metal contaminant ubiquitously found in water and groundwater. In this study, an optical biosensor for arsenite determination was developed by immobilization of crude arsenite oxidase (Aio) extracted from recombinant *E. coli*, in chitosan solution coated on triacetyl-cellulose membrane employing DCPIP as colour indicator. The arsenite oxidase (Aio) was successfully expressed and extracted from recombinant *E. coli* strain BL21 (DE3). The protein concentration and specific activity of the crude arsenite oxidase were determined. Expression of Aio was confirmed by SDS-PAGE. The crude Aio was also successfully immobilized in chitosan and coated on triacetyl cellulose membrane. The response time and dynamic range of the optical biosensor were optimized. The response time of the developed biosensor was 15 minutes. The amount of DCPIP reduced (ΔA) was inversely proportional to the arsenite concentration. Standard calibration curve for arsenite detection was achieved within the range of arsenite concentration from 25 µM to 200 µM. The maximum detection limit was determined to be 250 µM arsenite.

Keywords: Arsenite, biosensor, arsenite oxidase, recombinant, DCPIP

Abstrak

Arsenit adalah satu bentuk arsenik bukan organik yang sangat berbahaya kepada manusia. Ia adalah sejenis logam berat pencemar alam sekitar yang biasa terdapat dalam air dan air bawah tanah. Dalam kajian ini, satu biosensor optik untuk menentukan arsenit telah dicipta melalui immobilisasi enzim arsenit oksidase (Aio) mentah yang diekstrak daripada rekombinan *E. coli*, dalam larutan kitosan disalut pada membran triasetil-selulosa menggunakan DCPIP sebagai petunjuk warna. Arsenit oksidase (Aio) telah berjaya diekspres dan diekstrak daripada rekombinan *E. coli* daripada strain BL21 (DE3). Kepekatan protein dan aktiviti spesifik arsenite okidase mentah telah ditentukan. Ekspresi Aio telah disahkan melalui SDS-PAGE. Aio mentah telah berjaya diimobilisasikan dalam larutan kitosan dan disalut pada membran triacetil-selulosa. Masa tindak balas biosensor ialah 15 minit. Jumlah DCPIP dikurangkan (ΔA) adalah berkadar songsang dengan kepekatan arsenit. Keluk piawaian penentukuran untuk mengesan arsenit telah dicapai dalam julat kepekatan arsenit dari 25 µM 200 µM. Had pengesanan maksimum telah ditentukan pada kepekatan 250 µM arsenit.

Kata kunci: Arsenit, biosensor, arsenit oksidase, rekombinan, DCPIP

1.0 INTRODUCTION

Large number of recent studies have been focused on arsenic contamination due to its poisonous-metalloid that is naturally present in the environment mainly as a result of natural geologic activity (e.g., volcanic activity) and manmade resources (e.g., heavy industry, mining) [5]. According to the World Health Organization (WHO) (2012), arsenic is one of WHO's listed 10 chemicals that pose major public health concern. Arsenic is carcinogenic and toxic to human and animals.

Globally, arsenic contamination in drinking water is a major public health issue. Many South East Asia Region (SEAR) countries including Bangladesh, India, Vietnam, Nepal, Taiwan and PR China have reported the presence of arsenic ion of their contaminated ground water sources [8]. Thus, arsenic contamination through drinking water ultimately cause harmful and hazardous effect on the population [6].

The most common forms of arsenic that living cells (human and microorganisms) are generally exposed to are water soluble trivalent arsenite, As (III) or pentavalent arsenate, As (V) [14]. These species coexist as high as 74% As (V) and 98% As (III) from the total arsenic concentration in groundwater [9, 10, 12]. The toxicological effects of arsenic are dependent on its oxidation state and its chemical forms. As (III) is more toxic and mobile than As (V).

Sensitive detection for efficient removal of arsenic species is especially important. However, instrumental analysis such as ICP-MS and HPLC are costly, time consuming, require tedious sample pretreatment and is not generally portable for on- site monitoring. In contrast, the commercially available total arsenic kits, they are able to measure the total arsenic concentration. However, the kit does not identify the most toxic inorganic arsenic species, As (III).

Efforts have been put in to developing biosensor for the detection of total arsenic concentration, for example, Roberto *et al.,* (2002) [11] used green fluorescent protein and luciferase as reporter genes. Even so, not many studies focus on the detection of As (III), which is the most toxic form of the wide spread arsenic species. To date, only one form of arsenite biosensor has been developed [7]. This sensor uses molybdenum containing arsenite oxidase (Aio), which was obtained from the chemolithoautotroph NT-26, an arsenite oxidizing bacterium.

In this project, an optical biosensor was developed based on the immobilized crude Aio enzyme for detection of arsenite. Arsenite oxidase gene from *Thiomonas delicata* DSM 16361 [3] was cloned and expressed using *E.coli* system. Crude arsenite oxidase was further immobilized for the construction of biosensor strip. This provides an alternative method for detection of arsenite at higher concentration.

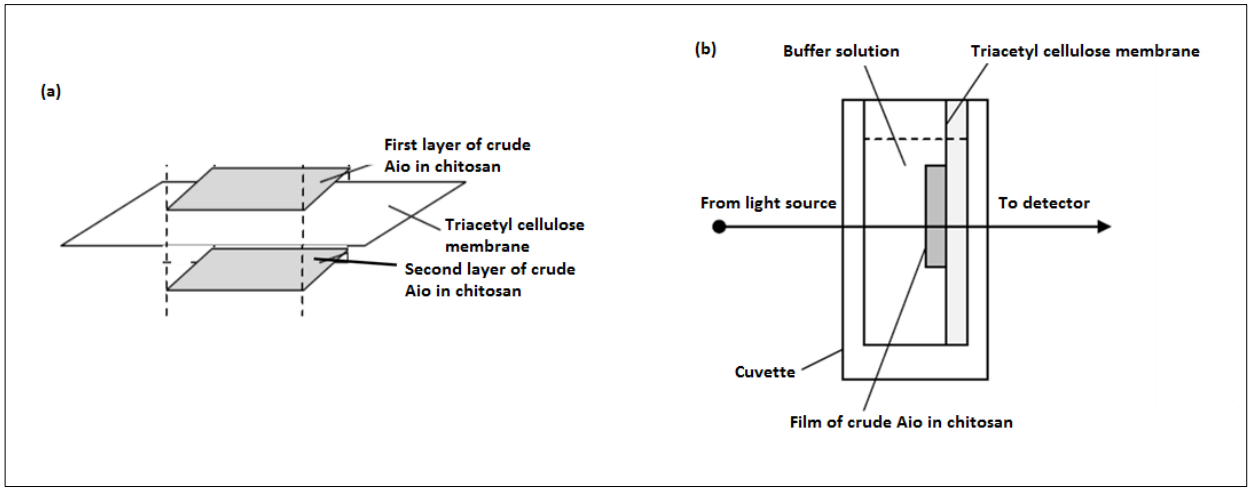


Figure 1 Schematic diagram of biosensor construction (a) Immobilization of crude Aio at triacetyl cellulose membrane (b) Set up of biosensor

2.0 MATERIALS & METHODS

2.1 Cloning and Expression of Recombinant Arsenite Oxidase

The full gene encoding for Aio from *Thiomonas delicata* DSM 16361 was cloned to pET-21(a) for expression in *E.coli* strain BL21(DE3). Expression was performed in LB broth aerobically using IPTG with final concentration of 40 µM and supplemented with 1 mM Na_2MoO_4 at 16°C for 16 hours.

2.2 Preparation of Crude Recombinant Arsenite Oxidase

Recombinant *E.coli* cell pellet was harvested and lysed by Bugbuster reagent (Novagen) according to

manufacturer's instruction and centrifuged for 20 minutes at 16,000 x g at 4°C for separation of crude lysate with unbroken cells. Crude enzyme was prepared fresh and used on the same day for biosensor construction.

2.3 Arsenite Oxidase Assay

Arsenite oxidase activity was assayed as previously described by Anderson *et al.,* (1992) [2]. Crude extract was diluted with 50 mM 2-[*N*-morpholino]ethanesulfonic acid MES buffer (pH 6.0). Aio activity was determined by measuring the reduction of 2,4- dichlorophenolindophenol (DCPIP) at OD600 in 50 mM MES containing 200 µM sodium arsenite. The enzyme activity was defined as µmol of DCPIP reduced/min/mg of protein. Protein concentration was determined by Bradford Protein Assay (Bio-Rad) using BSA as a standard according to manufacturer's instructions.

2.4 Biosensor Construction

The construction of this biosensor was divided to two parts, which is the immobilization support and biomaterial part. For the immobilization support, it was prepared based on the method by Ensafi and Amini [4] with some modification. The triacetyl cellulose membrane photographic film was cut into 9 mm x 30 mm and treated with commercial sodium hypochlorite to remove the coloured gelatinous layer and to increase porosity of the membrane. The preparation of the biomaterial was done based on the method used by Abdullah *et al*. [1] but with slight modification. Briefly, a homogenous mixture was prepared by mixing 5 mg/mL crude Aio with 2 % (w/v) chitosan at a volume ratio of 1.0 (v/v) in an eppendorf tube. The mixture was gently mixed for about 10 mins. 50 µL of the stock crude arsenite oxidase/chitosan solution was dropped onto the surface of film and smeared gently all over the film surface. After that, the same amount of the stock crude arsenite oxidase/chitosan solution was pipetted onto the other side of the film. The crude was immobilized on both of the film. Excess solution was removed by spinning the film at 1000 x g for 5 seconds and the film was dried for an hour at 4°C. All crude handling procedures were performed in cold condition.

2.5 Instrumentation and Measurement Procedure

All absorption measurements were conducted by using an ultraviolet-visible Spectrophotometer (Shimadzu). The membrane coated with crude Aio was soaked in 50 mM MES buffer solution pH 6.0 for 10 seconds to remove unbound enzymes, followed by washing with distilled water. Then, it was exposed to 3 mL reaction solution containing 50 mM MES buffer at pH 6.0 and 60 µM of DCPIP consisting of sodium arsenite ($NaAsO_2$) (µM). The response of the biosensor was studied at a fixed wavelength of 592 nm as described in the equation, Δ Abs (592 nm) = $I_{0\ min} - I_{n\ min}$. Absorption intensity of the biosensor at 0 min is represented by I_{0min} while $I_{n\ min}$ is the absorption intensity of the biosensor after incubated for 15 minutes.

3.0 RESULTS AND DISCUSSION

3.1 Expression of *Thiomonas delicata* DSM 16361 Arsenite Oxidase in *E.coli*

The cloning and expression of *Thiomonas delicata* DSM 16361 Aio was conducted in *E.coli* system. Without removing the twin-arginine translocation (TAT) signal peptide, Aio is a heterodimeric enzyme which comprises of large and small subunit to be enzymatically functional. As estimated by SDS-PAGE in Figure 2, size of large subunit (AioA) is approximately 91 kDa while small subunit (AioB) is around 21 kDa. Arsenite oxidase assay was carried out to confirm the expression with specific activity of 0.01 µM DCPIP reduced per min per mg protein.

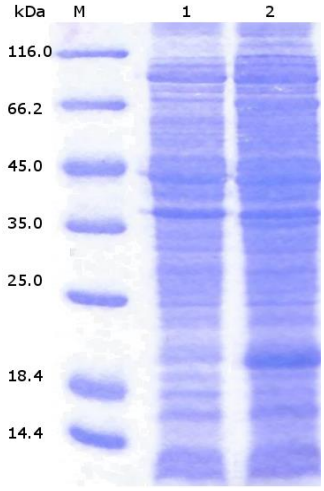


Figure 2 SDS-PAGE of crude lysate recombinant arsenite oxidase. M: Protein marker. Lane 1: Crude lysate without insert pET-21(a) vector as negative control. Lane 2: Crude lysate after IPTG induction

3.2 Biosensor Response

An optically transparent biosensor strip was constructed in this project by utilizing triacetyl celluloses and chitosan as immobilization support for crude Aio.

A series of experiments were performed in order to characterize the biosensor response. Arsenite is the substrate to establish the capability of the biosensor in terms of detection limit and response time. After the addition of arsenite, DCPIP and MES buffer to the biosensor strip, the response was monitored by measuring the changes in absorbance at 592nm which can be correlated to arsenite concentration. The mechanism of the DCPIP reduction during

oxidation of arsenite in the presence of biosensor strip enables the spectrophotometric monitoring of changes in absorbance of DCPIP.

Absorption spectra of the crude arsenite oxidase immobilized in a chitosan film in the presence of DCPIP was shown in Figure 3. It was noticed that, there was a slight shift in wavelength upon immersion of the optical biosensor into solution. This might be due to the different properties of chitosan or the tricetyl cellulose membrane itself. According to Abdullah *et al*., (2006) [1], chitosan might have little effect on the optical method. However it might not disrupt or change the reactivity of crude compared to other polymers due to its properties that are susceptible to chemical modification and are easily deposited on surfaces [16]. The absorption spectra of the enzyme biosensor were found to be consistent at 592 nm in the presence of different arsenite concentrations (Figure 4). Thus it can be concluded that the optimum response of biosensor was observed at wavelength of 592 nm.

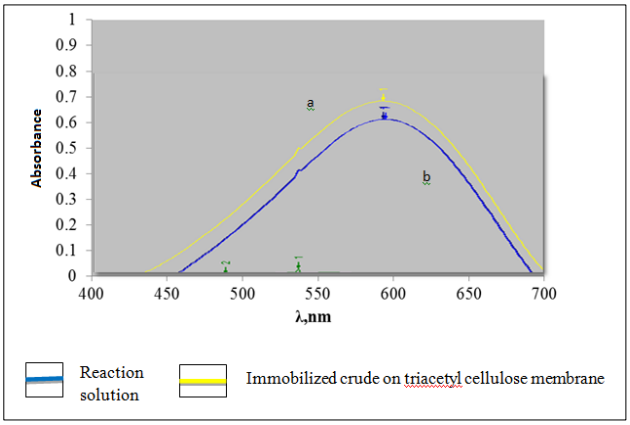


Figure 3 Absorption spectra represented by (a) Immobilized crude in 2% (w/v) chitosan in presence of 60 µM DCPIP and 50mM MES buffer. (b) Reaction solution containing 60 µM DCPIP and 50mM MES buffer without insertion of the membrane

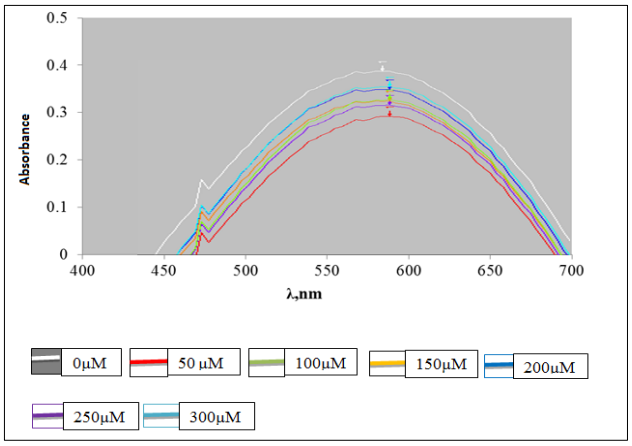


Figure 4 Absorption spectra of biosensor at different concentration of arsenite (0- 300 µM) in presence of 60 µM DCPIP and 50 mM of MES buffer at pH 6

3.3 Biosensor Sensitivity

Sensitivity of a biosensor is derived from its calibration curve. The slope of a linear calibration curve determines the sensitivity [15]. Standard calibration curve was plotted based on linear response at 15 min time reaction gained in Figure 5. The purpose of the calibration curve is to determine the relationship of the oxidation of arsenite by the optical biosensor towards different concentrations range of arsenite at their response time through reduction of DCPIP (ΔA). A linear response of the biosensor was achieved within the range of 25-200 µM arsenite.

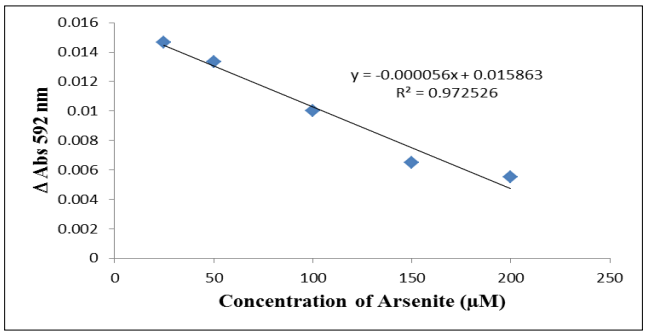


Figure 5 Calibration curve of the biosensor for arsenite detection [13]

4.0 CONCLUSION

The biosensor described in this preliminary work is a simple means for determination of arsenite with detection range from 25 to 200 µM. Nonetheless, further optimization has to be carried out to improve the performance of the biosensor for arsenite detection.

Acknowledgement

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