Designing A Bacterial Biosensor For Arsenic Detection In Water Solutions

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Abstract

In parallel to continuous development of increasingly more sophisticated physical and chemical analytical technologies for the detection of environmental pollutants, there's a progressively more urgent need also for bioassay which report not only on the presence of a chemical but also on its bioavailability and its biological effects. As a partial fulfillment of that need, there's been a rapid development of biosensors based on genetically engineered bacteria. Natural water was polluted by arsenic that known toxin and carcinogen leaded us to designing a whole cell bacterial biosensor to detect arsenic in liquid solutions.

Methods: In order to construct the biosensor, chromosomal *arsR* gene and its related promoter/operator from *Escherichia coli* strain *Bl21(DE3), gfp* gene as the reporter, plasmid pUC19 as the basic vector and different molecular and genetic engineering techniques such as PCR, gene cloning, etc. were applied to make the *Escherichia coli* strain *BL21(DE3)* act as the arsenic whole cell biosensor. Arsenic detection by using this biosensor was done by means of microscopy and fluorometery techniques. Strain *BL21(DE3)* responded mainly to As(III) and As(V) with the lowest detectable concentration being 5 µM during a 3-hours exposure and 1 and 3 µM respectively, with an 6-hours induction period.

Results: Our result demonstrate that the nonpathogenic bacterial biosensors developed in the present study could be useful and applicable in determining the bioavailability of arsenic with high sensitivity in contaminated water samples after further optimization, and they suggest a potential for its inexpensive application in field-ready tests.

Conclusion: Our success in designing and producing this biosensor, in addition to localizing this useful technology, will be a very large and effective step to improve the health status of drinking water, through to speed up detection of arsenic contamination of water resources. It can also attract lots of academic and industrial heads for research in this interesting field of science and establish an optimistic future in developing biosensor technology in our country.

Keywords *:arsR*, Arsenic Contaminant, Biosensor, GFP, Whole Cell Biosensor

1. Introduction

There are two common approaches to the monitoring of chemicals in the environment. The conventional one is based on chemical or physical analysis which is highly accurate and sensitive. So it is critical for regulatory purposes, and is necessary for understanding both the causes of pollution and the means for its potential remediation [1-3]. However, a complete array of analytical instrumentation necessary for such an extensive analysis is complex

and costly, and requires expert laboratories. In addition, such methodologies do not provide data regarding the bioavailability of pollutants, their effects on living systems, and their synergistic/antagonistic behavior in mixtures [4]. As a partial response to these needs, a complementary approach makes use of bioassays based on bio-systems and living organisms such as bacteria. These reporter bio-systems which are produced by means of genetic engineering tools and named as biosensors can be used to detect and even analysis chemical compounds and pollutants such as arsenic [5-8].

Arsenic contamination of soil and groundwater is a problem worldwide. According to WHO standards for safe drinking water (for most European countries and the united States, 10µg/L; elsewhere, 50 µg/L), it has been estimated that about 100 million people all over the world may be at risk of exposure to toxic and poisoned doses of arsenic [9].

The health effects of arsenic range from acute toxicity resulting from incorporation of arsenate as toxic phosphate analog, or arsenite inactivation of sulfhydryl containing proteins, to chronic effects including CNS damage, skin pigmentation abnormalities, and cancer [10]. Using bacterial biosensors, especially whole-cell type, start a new extensive field to develop rapid, low cost, easy and user friendly methods for arsenic detection and related diagnostic researches topics [11-13].

In the present study, we describe the construction and testing of a GFP whole-cell biosensor for the measurement of bioavailable arsenic. The sensor plasmid, designated as pUARG, is based on the expression of the *gfp* gene under the control of the *ars* promoter and the *arsR* gene of *E.coli* strain *BL21(DE3)*. Despite all the limitations, our simple arsenic whole-cell biosensor which could response to arsenic presence in water solutions in acceptable concentration range.

2. Material and Methods

Plasmid construction: Plasmid and *E.coli* strains, constructed and used in this study, were listed in Table 1, with their abbreviations and descriptions. The O/P region and the entire *arsR* gene were amplified by polymerase chain reaction (PCR) from fresh *E.coli BL21(DE3)* culture which used as template through colony PCR. Oligonucleotide primers used for PCR were designed based on the nucleotide sequence of *E.coli BL21(DE3)* DNA deposited at GenBank accession number AM946981, incorporated *Hind*III and *BamH*I restriction endonuclease cut sites to promote subsequent ligation with pUC19 vector. *pfu* DNA polymerase (Fermentas) was used in PCRs. The resulting 506 bp fragment was digested with *Hind*III and *BamH*I enzymes and ligated with T4 DNA ligase (Fermentas) into pUC19 (previously digested with the same enzymes). The approved 2.89 kb construct (pUARS) was transformed into the *E.coli DH5α* (replicating host) and *E.coli BL21(DE3)* (expressing host).

pIVEX-GFP was used as the PCR template to amplify the *gfp* gene. The resulting PCR fragment was ligated to *Pst*I and *Eco*RI sites next to *arsR* in pUARS after pre-digestions with *Pst*I and *Eco*RI enzymes. The final 3.609 kb construc was transformed into the *E.coli DH5α* and *BL21(DE3)*.Colony selections were done using ampicilin in this study. The direction of the cloned genes and fragments were confirmed by restriction digest and PCR.

All the molecular methods were performed regard to "Molecular cloning, A laboratory manual" [14].

Culture and arsenic assay conditions: *E.coli* strains were grown in Luria-Bertani (LB) medium at 37°C in an orbital incubator (Biotek). *E.coli* strains carrying plasmids constructed from pUC19 were selected with ampicilin (100µg/ml) respectively. For arsenic sensitivity tests, cultivation in test tubes was started with 100-fold dilution of freshly prepared overnight culture of *E.coli* strains harboring pUARS in 5 ml medium. To monitor cell growth, absorbance at 600 nm (A_{600}) was measured with the spectrophotometer (OPTIZEN). When the A_{600} of culture reached 0.4-0.6, cells diluted 100-fold in LB broth and 0.1 ml of these dilutions were spread on LB agar palates which contained 100µg/ml ampicilin and various concentrations of arsenite and arsenate ions. Plates were incubated overnight at 25-30°C. The colonies were counted after 18 hours [9].

Microscopy: In order to arsenic biosensing assays, a single colony of *E. coli* strain *BL21(DE3)* harboring pUARG was grown overnight in Luria-Bertani (LB) medium supplemented with 100 µg/ml of ampicilin at 37°C. The overnight culture was diluted 100-fold in fresh LB medium supplemented with 100 µg/ml of ampicilin and incubated at 37°C in an orbital shaker at 220 rpm until the optical density at 600 nm reached 0.6.

Various concentrations of As(III) and As(V) were added to bacterial cultures.

Assay mixtures contained 2 ml of diluted cell suspension and 2 ml fresh LB containing As(III) and As(V). Final concentration of arsenic in assay mixtures would be: 1.0, 3.0, 5.0, 10.0, or 20.0 µM arsenic. Assays were incubated at 30 °C in a rotary shaker (190-220 rpm). For GFP measurements, culture samples of 200 µl (after 3 h) and 100 µl (after 6 h) were centrifuged for 2 min at 15000*g*, and the supernatant decanted. The cell pellet was washed 2 times with 500 µl of PBS and resuspended in an

appropriate amount of phosphate buffer saline (PBS) (between 10 and 50 µl, depending on the amount of cells) [9,15].

One microliter of cell suspension was transferred to a microscope slide, and the fluorescence of individual cells was determined by epifluorescence microscopy [16]. *E.coli* strain *BL21(DE3)* which was harboring pIVEX-GFP was used as the positive control by using IPTG as the inducer for promoter T7 and expression of GFP. Also *E.coli BL21(DE3)* containing plasmid pUC19 was used as negative control. Cells were viewed using an epifluorescent microscope (Zeiss upright fluorescent microscope) equipped with a 100 W mercury arc lamp and a filter block fitted with a 385–425 nm excitation filter, a 450 nm long pass dichroic mirror, and a 500–540 nm emission filter [15].

Fluorometery: The transcriptional activity of the biosensor was estimated by the measurement of the GFP fluorescence of cells grown in LB medium containing a range of different arsenic ions. Cell growth was monitored by the measurement of optical density at 600 nm with a spectrophotometer. The fluorescence of GFP producing cells that were grown in culture was measured using a Fluorometer (JASCO FP-6200). The excitation wavelength of the fluorometer was set at 490/10 nm, and the emission wavelength was set at 530/10 nm. *Escherichia coli BL21* carrying pUC19 was used as the baseline sample to zero the instrument.

We considered the effects of cell growth on signal and noise intensities. The total fluorescence intensities (arbitrary fluorescence units, AFU) were divided by the A_{600} of subcultures (Without subtracting and any background values. Signal and noise were defined as AFU/A600 or AFU in the presence and absence of As, respectively. The signal-to-noise ratios were defined as the ratio of signal (AFU/A_{600}) to noise (AFU/A_{600}) or the ratio of signal (AFU) to noise (AFU) [15].

Raw fluorescence values were expressed in the instrument's arbitrary relative fluorescent units (AFU). The specific fluorescence intensity (SFI) is defined as the raw fluorescence intensity expressed in relative fluorescence units divided by the optical density at 600 nm measured at each time point. At least triplicate measurements were obtained for each sample after 3 and 6 hours of exposure [15].

3. Results

Through the arsenic sensitivity test, E.coli strains harboring pUARS were spread on prepared agar plates containing various concentrations of arsenic and arsenate ions. The colonies were counted after 18 hours incubation. As it can be seen obviously in Figure 1, increasing the concentrations of arsenic compounds leaded to reduction in size and number of colonies.

In order to arsenic biosensing with our GFP biosensor, expression of green fluorescent protein under control of ars promoter was induced by adding various concentrations of As (III) and As (IV) for 3 and 6 hours at 30°C in LB medium. Bacterial cells were visualized by using an epifluorescent microscope after 3 hours exposure to arsenite. As it can be seen in Figure 2, in presence of 10µM arsenite, the number of fluorescent bacteria and the intensity of produced fluorescent were obviously more than other arsenite concentrations.

Number of fluorescent bacteria and the intensity of produced fluorescent were increased due to rise of arsenite concentrations in 0-10µM. However, in presence of 20µM arsenite, an obvious drop was observed in number of fluorescent bacteria and the intensity of produced fluorescent. Fluorometery results which were summarized in Tables 4 & 5 and Figures 3 & 4, showed that AFU and SFI increased due to enhancement of arsenite ions concentrations up to10 µM. However in concentration of 20 µM of arsenite, a reduction could be observed in AFU and SFI while the results of the similar assays which various arsenate concentrations were applied, did not show the reduction in AFU or SFI even in arsenate concentration of 20 µM. Also, as it was predicted, measured AFU and SFI were certainly less in all the concentrations of arsenate compared to those of arsenite.

4. Discussions

Although biosensor technology is getting developed all over the world, in our country, designing and production of biosensors are already on the primary steps and according to our studies, there are not any publications that have been reported to use these devices, especially whole-cell types, in environmental analyses.

According to the reports- by research groups in Environmental Health, Hamedan University of Medical Sciences - arsenic contamination has been observed in drinking water supplies in Hamadan as well as other parts of the country especially the northern areas, in this study, by means of *arsR* gene (from *E.coli* strain BL21), plasmid pUC19 (as the vector) and GFP protein(as the reporter) and utilize genetic engineering techniques, we have tried to design and produce a whole-cell bacterial biosensor that has potential to detect presence of arsenic specially in liquid samples.

Based on our readings, there is not any report to use such a gene construction to design arsenic biosensor.

Several bacterial biosensors based on transcriptional fusions between arsenic-inducible promoters and

reporter systems have been described [17-19]. These biosensing systems are based mostly on the resistance mechanism that is encoded by the *ars* operon found on the *E.coli* plasmid R773 [18,20]. In contrast, Tauriainen et al. employed a different regulatory unit that was derived from the *ars* operon of plasmid pI258 from *S. aureus* [21].

In order to construct a whole cell bacterial biosensor to detect arsenic, in our study, we were very interested to use plasmid R773 as the *arsR* gene and its related operator/promoter. However the plasmid R773 was unavailable for us. So we decided to select one of the similar sources of the gene that was accessible, chromosome of E.coli BL21(DE3).

As it has been shown in Table 6, Bioassays with subsequently reengineered *E.coli* sensor/reporters with bacterial and firefly luciferase were capable of detecting lower concentrations of arsenite that are at or below the most common drinking water standard of 10µg As/L [12]. Bioassays with very low detection ranges for arsenic in solution (10–50µg/L) were also reported using *E.coli* sensor/reporter cells expressing β-galactosidase. Assays with these cell lines display a linear detection range for arsenic concentrations in solution either less than 10µg/L or less than 50µg/L [12]. Assays with *E.coli* cells expressing GFP as a reporter protein display a wider arsenic detection range than the β-galactosidase reporters. The GFP reporter assays have a linear detection range for arsenic in solution ranging from 1 to 100µg/L, from 8 to 47µg/L and from 8 to 234µg/L, and from 78 to 390µg/L [12]. Cytochrome c peroxidase has also been utilized as a reporter to produce an *E.coli* arsenic biosensor. Bioassays with cytochrome c peroxidase reporter strains showed linear detection ranges for arsenic ranging from 4 to 20 or 30µg/L and from 20µg/L to 5 mg/L, as well as in ranges greater than 1 mg/L, dependent on genetic differences of the reporter strain [12]. The development of arsenic bioassays using *B. subtilis*, *S. aureus*, and *R. palustris* biosensor cells has occurred to a lesser extent. Only two *B. subtilis* sensor/reporter strains for arsenic have been created; one which expresses firefly luciferase and the other β-galactosidase.

Bioassays with the firefly luciferase reporter achieved a linear detection range for arsenic of 257–7,800µg/L [22], whereas with the β-galactosidase reporter a linear detection range for arsenic of 22–7,800µg/L was found [23].

In our study the range of arsenic detection (As(III): 130 µg/L-2.6 mg/L and As(V): >312 µg/L) is obviously limited compare to mentioned studies. This phenomenon could be the result of our promoter/operator region or *arsR* gene. Through bioinformatic analyses, we focus on *arsR* genes and ArsR proteins from plasmid R773 and *Escherichia coli BL21(DE3)* chromosome.

It was shown a high level of similarity (85.5%) between these two *arsR*, but in comparative view it can easily be observed that in helix-turn-helix motif of *arsR* from R773, there are two methionine residues which are absent in chromosomal *arsR* gene from *E.coli BL21*. ArsR protein from *E.coli BL21* has threonine and leucine instead of methionine in sites 38 and 53. Since ArsR is one of the proteins with winged helix topology, it could not be weird that difference in amino acids composition related to Helix Turn Helix (HTH) motif results in different level of sensitivity expression to arsenic compounds between *arsR* genes from R773 and *E.coli BL21* through trace on conformation of protein and its reaction with DNA [24,25]. Obviously, more practical analysis such as directed substitution mutations is necessary to prove our suggestion about the methionine residues role.

In this study, the fluorometery results showed that the intensity of GFP increased with increasing amount of As(III) to a concentration of 10 mM. When the As(III) concentration increased to levels greater than 10M, the fluorescence started to decrease. This might be caused by the toxicity of As(III) ions to the bacterial cells. Since the biosensor cells contain the arsenate reductase enzyme, they also respond to arsenate. However, when the same batch of cells was tested with arsenite and arsenate in the same concentration range, the cells responded with lower light output for arsenate than for arsenite(approximately two fold lower in concentration of 10µM). The reason for this difference may be that the rate of arsenite production from arsenate by the arsenate reductase does not follow the same kinetics as the interaction of arsenite with ArsR or the efflux rate. These results are similar to those from other papers [16].

Although our biosensor cannot have such a powerful performance compare to advanced arsenic biosensors because of all mentioned reasons, however, this simple biosensor, despite all limitations, will be able to response to lots of needs in arsenic contamination problems in field studies and just with some optimizations, its specificity and sensitivity could rise up to those of confirmed arsenic biosensors, promisingly.

Using of bacterial arsenic biosensors especially Whole-cell type, have many advantages over conventional methods. The best and most valuable advantage of this type is their ability to identify bioavailable parts of the total amount of arsenic that leads to precise assays and more accurate estimates. In the presence of arsenic, whole-cell biosensors

Table 1: Plasmids and E.coli strains constructed and used in this study

Plasmids or strains	Designations or genotypes	Descriptions	Source
Plasmids			
pUC19		Basic vector, amp^R gene	Fermentas
pIVEX-GFP		Source of gfp gene, amp^R gene	Pastaur institute
pUARS	pUC19:P_{ars}-arsR	arsR expression from P_{ars} by arsenic induction	This study
pUARG	pUC19:P_{ars}-arsR-gfp	arsR and gfp expression from P_{ars} by arsenic induction	This study
Strains			
E.coli DH5α	F– *gyrA96* (Nalr) *recA1 relA1 endA1 thi-1 hsdR17* (rk–mk +) *glnV44 deoR* Δ*(lacZYA-argF)U169* [ϕ80dΔ*(lacZ)M15*]	Replicating host	Razi institute
E.coli BL21(DE3)	F- *ompT hsdSB(rB- mB-) gal dcm (DE3)*	Source of O_{ars}/P_{ars} region, source of arsR gene, expression host	Cinnagen

Table 2: Whole cell - GFP bacterial biosensors for arsenic measurements

Bacterial sensor-reporter strain	Method of As detection	Utilization	Reported range of As(III) detection	References
***E.coli* (pIRC140)**	Green fluorescent protein	96-well plate	1–100µg/L	9
***E.coli* DH5α (pPR-arsR)**	Green fluorescent protein	Microscope slide	8–47µg/L	9
***E.coli* DH5α (pPR-arsR-ABS)**	Green fluorescent protein	Microscope slide	8–234µg/L	6
***E.coli* AW10 (pSD10)**	Green fluorescent protein	Microfluidics device	78µg/L–390 mg/L	26
***E.coli* BL21(DE3) (pUARG)**	Green fluorescent protein	Microscope slide, Fluorometry	As(III): 130 µg/L-2.6 mg/L As(V): >312 µg/L	This study

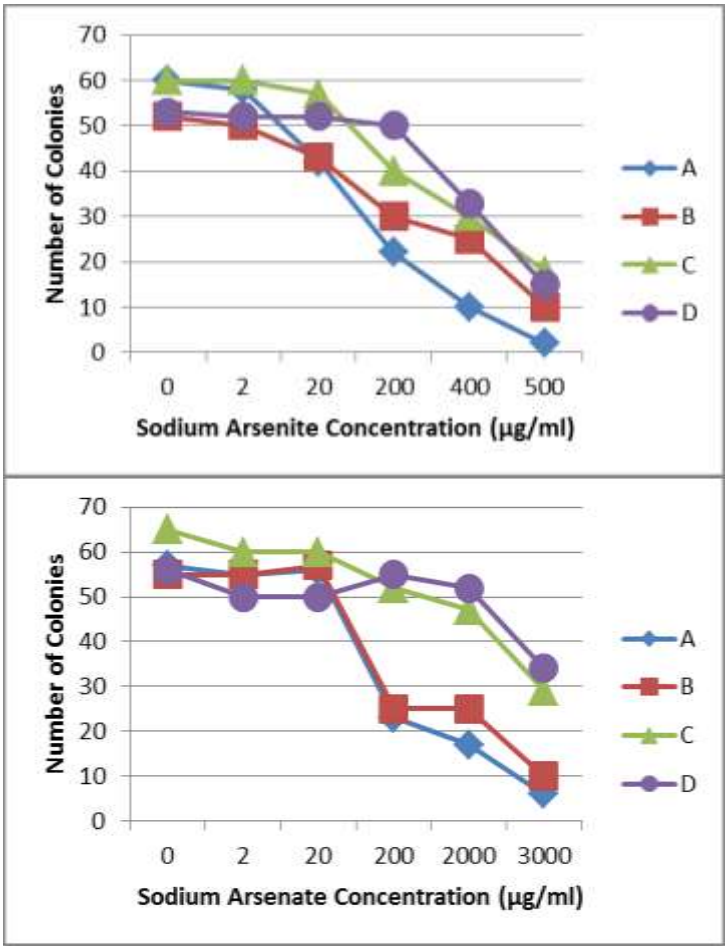

Figure 1: Arsenic sensitivity tests on solid media. Log-phase cultures of *E. coli DH5α* and *BL21* harboring plasmids were spread on petri dishes that contained the indicated concentrations of sodium arsenite (up) and sodium arsenate (down), as described in Materials and Methods. Colonies were counted after 18 h . A: *E.coli DH5α*:pUC19, B: *E.coli DH5α*: pUARS, C: *E.coli BL21(DE3)*: pUC19, D: *E.coli BL21(DE3)*: pUARS

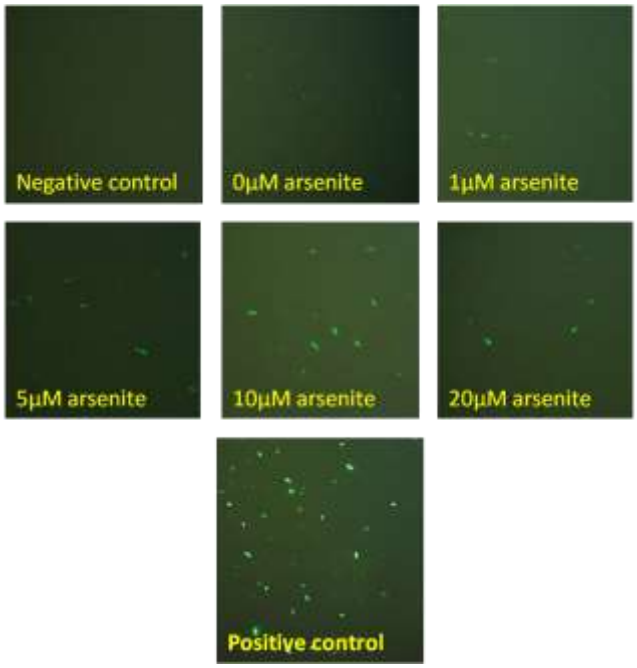

Figure 2: Fluorescence of biosensor exposed to As(III)

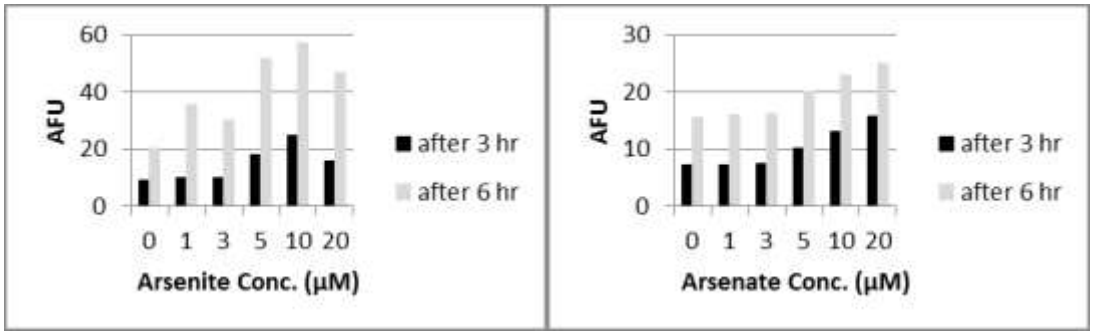

Figure 3: Fluorescence response of *E. coli BL21(DE3)* suspensions carrying pUARG after induction of GFP in the presence of arsenite (left) and arsenate (right). Fluorescence measurements were performed in triplicate (average values shown) after 3 and 6 hours.

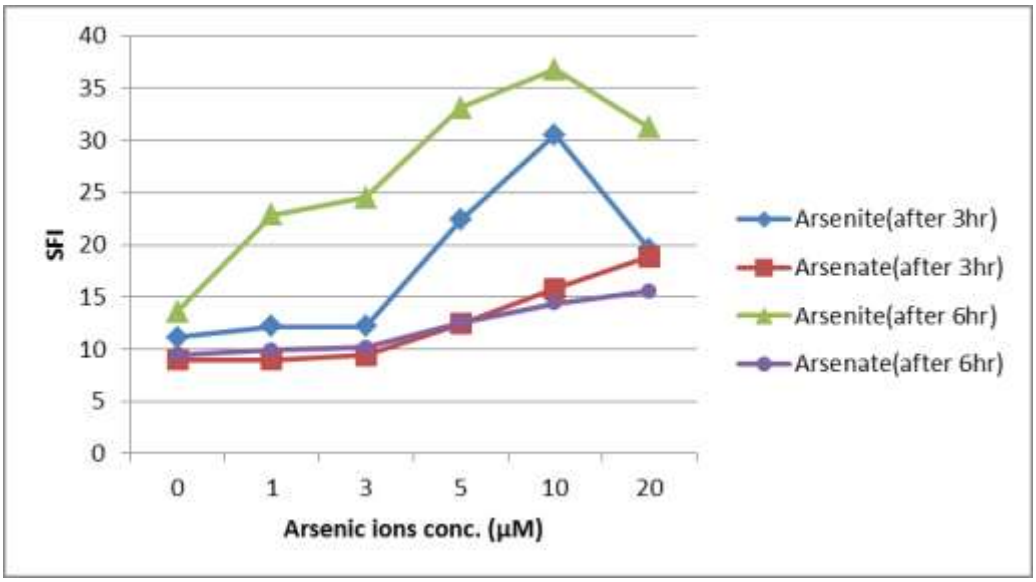

Figure 4: SFI measurements. The specific fluorescence intensity (SFI) is defined as the raw fluorescence intensity expressed in relative fluorescence units divided by the optical density at 600 nm measured at each time point

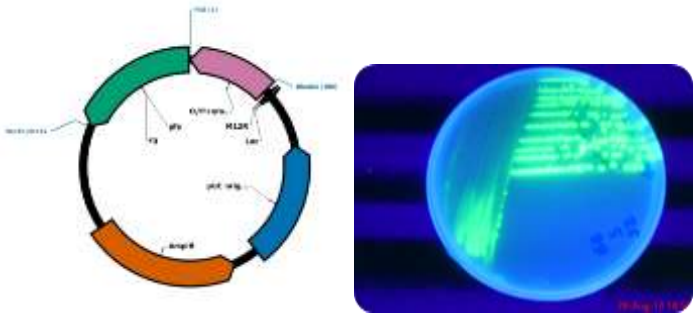

Figure 5: Cloning and construction of biosensor plasmid (right). GFP expression in biosensor plasmid under UV light (left)

create clear responses through physiologic pathways which are easy and fast to be identified. So biosensors can be taken into account as the real time diagnostic systems.

In recent 30 years, design and production of biosensors for contaminants and toxin detection in environment have been increasing dramatically. Since that time, eminent companies producing biotech products, in cooperation with environmental protection institutions, pharmaceutical companies and food industries have become the biggest supporters of the design, production and development biosensors especially the whole-cell types.

Our success in designing and producing this biosensor, in addition to localizing this useful technology, will be a very large and effective step to improve the health status of drinking water, through to speed up detection of arsenic contamination of water resources. It can also attract lots of academic and industrial heads for research in this interesting field of science and establish an optimistic future in developing biosensor technology in our country.

It is hoped that further study on the capabilities of this biosensor and enhancement of its accuracy, leads to its production in industrial scale. So that in addition of water, it will be able to detect the exact quality and quantity of arsenic present in various samples such as body fluids. Even more, besides detection, it will be able to remove arsenic contamination from the sampe

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