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# Development of Bacterial Biosensor using gfp and lacz reporter genes for Arsenite detection in potable water samples from Gangetic Delta

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## Abstract

Arsenic has long held a position of ambiguity with regard to its activity in biological systems. Arsenic contamination in deeper levels of groundwater, which became a high-profile problem in recent years due to the use of deep tube wells for water supply in the Ganges Delta. It causes serious arsenic poisoning to large numbers of people. Recent study found that millions of people in more than 70 countries are probably affected by arsenic poisoning of drinking water. Detection of arsenic levels in potable water can be done with development of whole cell bacterial biosensor using reliable and robust genetic engineering approaches. Isolated Pr-ABS-*arsR* gene was cloned in pUC18 and confirmed by agarose gel electrophoresis. Similarly, reporter gene *gfp* was also isolated and cloned in pUC18-Pr-ABS-*arsR* construct. To reduce the background expression second copy of ABS inserted into pUC18-Pr-ABS-*arsR*-*gfp* and pUC18-Pr-ABS-*arsR*-ABS-*gfp* was obtained. This modified construct was then transformed into *E.coli* DH5α. These transformed cells can be developed as whole cell biosensor and applied in field. A physical gadget for measuring arsenite was tested in both lac Z and *gfp* based whole cell biosensor.

**Key words:** Arsenite, ARS binding protein, whole cell biosensor, *gfp, lac Z*

## Introduction

Industrialization and new technologies have not only made life more convenient for humans, but have also created various environmental problems, potentially poisoning living organisms, including people. Heavy metals from industries bioaccumulate in the aquatic food chain to the extent that the consumption of fish by birds and animals[8]. Organic compounds, originating from the widespread use of petroleum products, are highly toxic and causes concern about soil and drinking-water quality[1]. Conventional chromatographic methods for evaluations are both expensive and technically complicated. They tend to overestimate the bioavailability of the pollutants, since metals and xenobiotics often exist in an insoluble form in the environment[5,13]. The linking of biological component with a microelectronic device or system to enhance the rapid, low level accurate detection of various substances like body fluids, water and air[2]. In recent years, bacterial whole-cell biosensors have been developed as tools to detect and quantify the toxicity of samples from different environments. They are all living cells producing a measurable gene product in the presence or absence of the investigated compounds. In all cases, detection of various xenobiotic compounds by the bacterial biosensors described in all other review requires direct interaction between the compound measured and the biosensor, which therefore gives a direct measurement of the impact on living organisms. In

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addition, we hope to show that the use of biosensors is not limited to risk assessment of pollutants, but also has evolved to constitute a valuable tool in studying microbial ecology and gene expression in complex environments.

## Arsenic Forms and Toxicity

Arsenic in the atmosphere was from sources, such as volcanoes (gas fumes), microbes, and human activities alike burning fossil fuels.

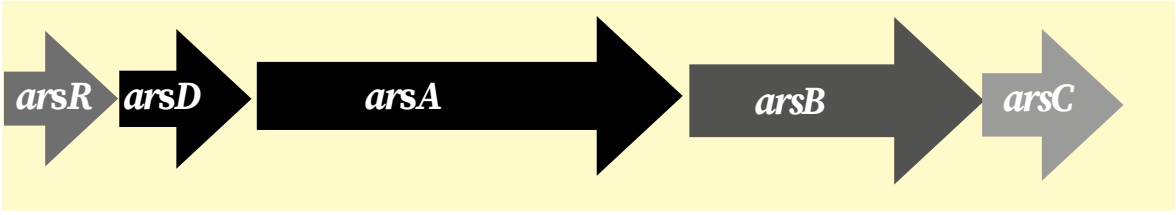


Fig.1 Ars operon of E.coli R773

The toxicity of arsenite is due to the formation of strong bonds with functional groups, such as the thiols of cysteine residues and the imidazolium nitrogens of histidine residues from cellular proteins. In the case of arsenate, its toxicity is the result of the mimetic effect of arsenate ($AsO_4^{3}$) and phosphate ($PO^{3-}$) which affects global cell metabolism[15]. In the present work, the fabrication of whole cell biosensor is carried out and used the green fluorescent protein as reporter protein (*gfp* gene).

## Materials and Methods

### Plasmid Isolation

The plasmid pBGD 23 was isolated from *E.coli* R773 by alkaline lysis method. Agarose gel electrophoresis was adopted for elution of the gene of interest.

Table 1. PCR specified conditions

| Phase | Temperature (°C) | Time (seconds) |
|---|---|---|
| Denaturation | 94 | 30 |
| Annealing | 55 | 30 |
| Extension | 72 | 30 |
| Final extension | 72 | 300 |
| Final hold | 4 | - |

### Amplification of Repressor and Reporter Gene

Amplification of *arsR* gene with its promoter and *arsR* binding site with its primer's PCR. The primers for *ArsR*are**5` ccc tttcgtcttc*aacgttt*ccaag 3`**; 129 bp upstream of the start of *arsR* and introducing a *Hin*dIII site and *ArsR* rev **5` aacata*tgaatt c*ag gcaaatttttag 3`**, covering the stop codon of *arsR* and introducing a unique *Eco*RI site for amplifying *arsR* along with its promoter. The *gfp* gene present in the plasmid p*GFP*uv (from Clone tech. corporation, CA, USA) was amplified. The amplified reporter gene was cloned in downstream of sensing part (*arsR)* at appropriate restriction site in the MCS of pUC18-*arsR*. The resulting plasmid consists of *arsR* and *gfp* genes. Agarose (1%) gel electrophoresis was carried out for eluting repressor and reporter gene amplified through PCR.

### Restriction digestion of PUC18 vector

Eluted *ArsR*andpUC18 vector was added with restriction enzyme buffer and one unit of the BamHI restriction enzyme. The reaction volume was made up to 40µl with sterile water. Then contents were centrifuged and incubated at 37°C for 2 hr. The reaction was terminated by adding phenol/EDTA and the sample was frozen immediately.

### Ligation of Pr-ABS-ARSR gene into PUC18 vector

Restricted *Pr-ABS-arsR* and pUC18 vector were ligated by $T_4$ DNA ligase and the reaction volume

was made up to 20µl. It was mixed thoroughly and incubated at 12°C for overnight.

### Transformation of PUC18-Pr-ABS-arsR construct into E.coli DH5α

*E.coli* competent cells and pUC18-Pr-ABS-*arsR* plasmid was added and mixed. It was then incubated in ice. A heat shock for 2 min was given at 42°C and again incubated in ice. The transformed cells (100 µl) were plated on selective plates (Luria agar containing 50 µg/ml of ampicillin) and incubated for 16 to 17 hr at 37°C.

### Insertion of second ABS in constructed plasmid

Background expression can be reduced by the insertion of second copy of ABS to the constructed plasmid. The steps involved are a) Amplification of ABS with given primer sequence in polymerase chain reaction b) Restriction with Eco RI enzyme and ligation using $T_4$ DNA ligase into constructed plasmid c) Transformation into *E.coli* DH5α cells

### Restriction with EcoRI enzyme and ligation using $T_4$ DNA ligase into constructed plasmid

The restriction pattern was followed same as 3.1.6, with restriction enzyme as EcoRI and its corresponding buffer. The amplified and eluted ABS sequence was then ligated with pUC18-Pr-ABS-*arsR-gfp* construct. Transformation of pUC18-ABS-*arsR*-ABS-*gfp* was done.

### Amplification of response and reporter gene

The amplification of the repressor gene *arsR* and the reporter gene *gfp* was confirmed by agarose gel electrophoresis against marker DNA of 1kb size. The approximate size of insert gene was 0.7 kb. A Prominent band near 0.7 kb was observed under uv transilluminator (Genei, India). The amplified reporter gene was confirmed similarly which shows the size was approximately 1.1 kb.

### Restriction digestion and ligation of Pr-ABS-arsR and gfp to PUC18

The restriction digestion of Pr-ABS-*arsR* and vector pUC18 using BamHI was confirmed by the presence of 0.7 kb and 2.9 kb linearized bands on the agarose gel. Similarly, *gfp* gene also restricted using Hind III enzyme and confirmed through 0.6 kb size band.

### Confirmation of presence of second copy of ABS

The constructed plasmid was modified by inserting second copy of ABS to reduce background expression and confirmed through PCR. The ABS amplify the region between two ABS inserts and confirmed through agarose gel. The band obtained near 0.8 kb size as well as 0.3 kb showed the two copies of ABS in the modified construct pUC18-Pr-ABS-*arsR*-ABS-*gfp*.

### Screening of recombinants

Through blue and white colonies growth was observed distinctly in $10^{-5}$ diluted plates. The white colonies were separated and patched in LB media with ampicillin (50 µg/ml). The transformants were isolated for modified plasmid and insertion of response and reporter gene was confirmed by restriction digestion by Hind III. Prominent band near 1.8 kb shows the presence of both genes in agarose gel electrophoresis under UV transilluminator.

### Arsenite detection assay

The arsenite measurement assay procedure was carried out for various river water samples and found to detect higher concentration of arsenite than that of ICP-OES16. The ICP – OES found to quantify arsenite of 2 ppm concentration. The detection of arsenite was in the range of 7 µM to 50 µM for whole cell biosensor containing gfp as reporter gene. The fluorescence obtained for standard concentration of arsenite was shown in the figure below. Constructed pUC18 plasmid which can detect the arsenite in the potable water sample. Also, a physical gadget was fabricated to test the arsenite level of contamination.

## RESULTS AND DISCUSSION

Arsenic has long held a position of ambiguity with regard to its activity in biological systems. Arsenic contamination in deeper levels of groundwater, which became a high-profile problem in recent years due to the use of deep tubewells for water supply in the Ganges Delta. It causes serious arsenic poisoning to large numbers of people. Recent study found that millions of people in more than 70 countries are probably affected by arsenic poisoning of drinking water. Detection of arsenic levels in potable water can be done with development of whole cell bacterial biosensor using reliable and robust genetic engineering approaches. Isolated Pr-ABS-arsR gene was cloned in pUC18 and confirmed by agarose gel electrophoresis. Similarly, reporter gene gfp was also isolated and cloned in pUC18-Pr-ABS-arsR construct. To reduce the background expression second copy of ABS inserted into pUC18-Pr-ABS-arsR-gfp and pUC18-Pr-ABS-arsR-ABS-gfp was obtained. This modified construct was then transformed into E.coli DH5α. These transformed cells were developed as whole cell biosensor and assayed for the presence of arsenite. The developed sensor emits fluorescence which is exploited to show presence of arsenite.

The developed paper strips were analyzed for the blue color development with the response to the arsenite concentration with the substrate addition of 10µl of 2mg/ml X-gal stock, the blue color development for minimum concentration of arsenite in standard arsenite stock solution was observed from 6.5µM.

The sensor was developed as portable, flexible physical gadget with transmitter, optic fiber and receiver elements for quantification of arsenite. The bridge board circuit was well worked out and shown as figure below. This was transferred into proto model with the help of PCP assistance which shows deflection in voltage of multimeter. The fabrication of the biosensor as given in the circuit was done. UV or Infrared transmitter is one type of LED which emits ultraviolet or infrared rays generally called as UV/IR Transmitter. Similarly, receiver is used to receive the rays transmitted by the transmitter. One important point is both transmitter and receiver should be placed straight line

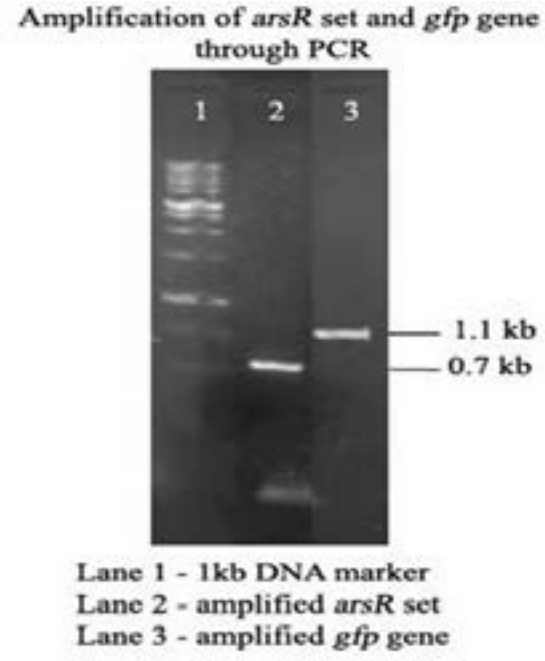


Fig.2 Agarose gel electrophoresis of amplified arsR gene and gfp gene.

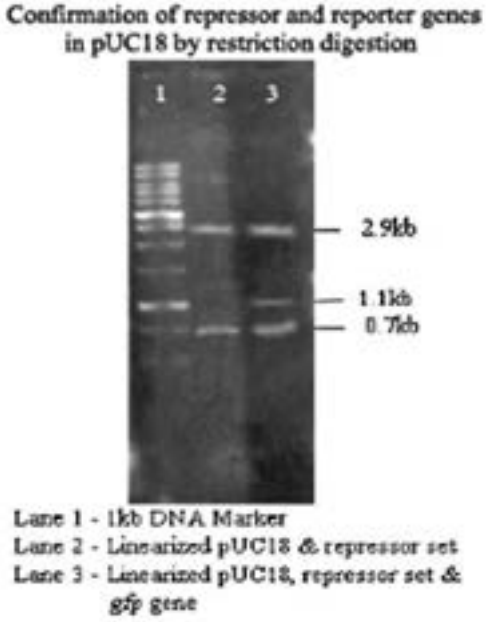


Fig.3 Agarose gel electrophoresis of repressor and reporter gene pUC18 by restriction digestion

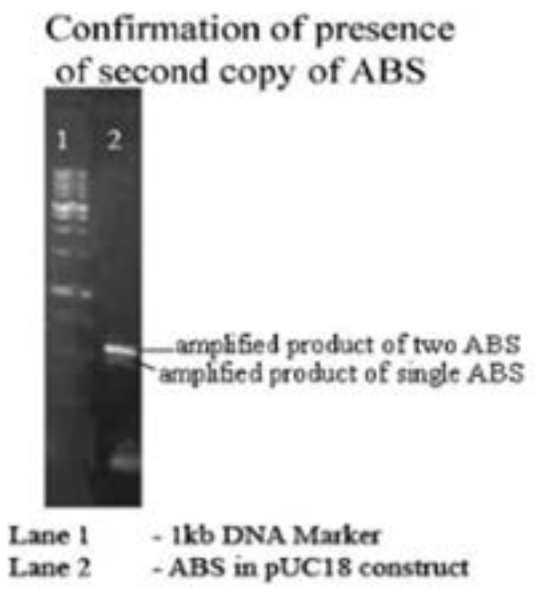


Fig.4 Agarose gel electrophoresis of second copy of ABS

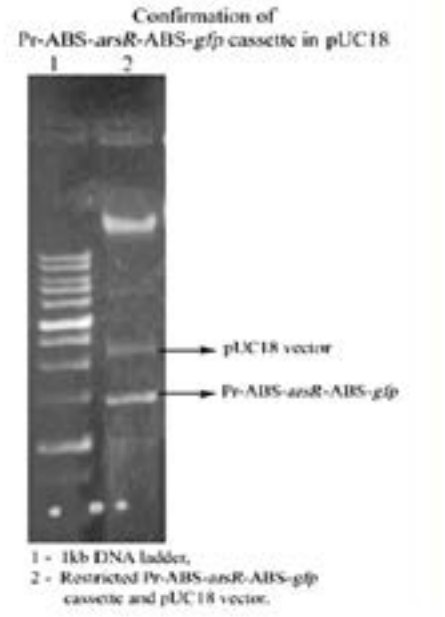


Fig.5 Agarose gel electrophoresis of Pr-ABS-arsR-ABS-gfp cassette

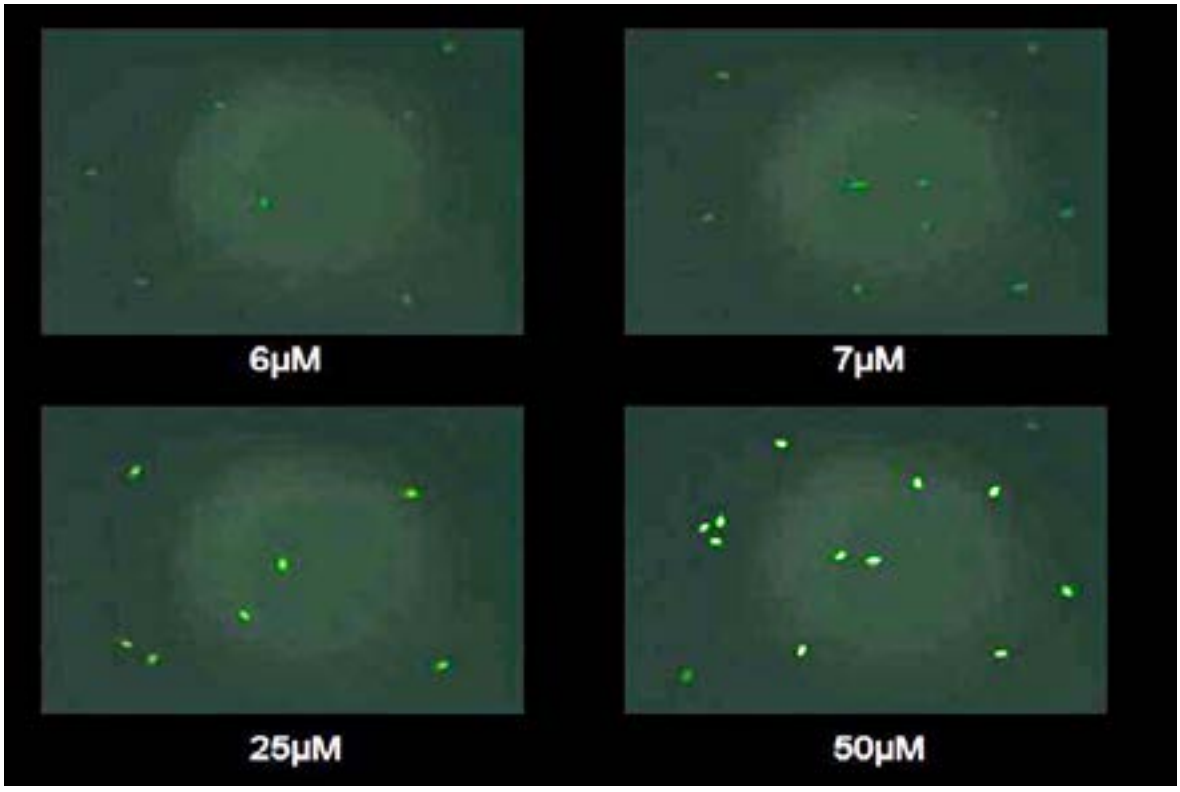


Fig.6 Detection of Arsenite at different ranges

to each other. Whenever the transmitted signal is high, the transmitter LED is conducting and it passes the rays to the receiver. The receiver in turn connected with comparator. The comparator is constructed with LM 358 operational amplifier. In the comparator circuit the reference voltage is given to inverting input terminal. The non-inverting input terminal is connected to receiver. When interrupt the emitted rays between the transmitter and receiver, the receiver is not conducting. So, the

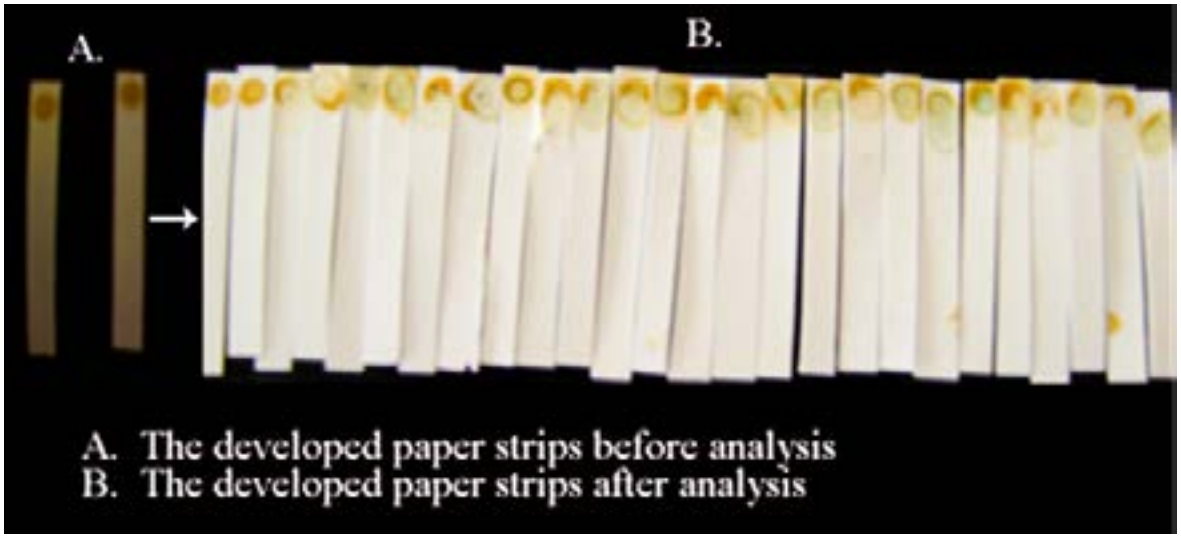


Fig. 7 Development of portable whole cell biosensor

comparator noninverting input terminal voltage is higher than inverting input. Now the comparator output is in the range of +5V. This voltage is given to microcontroller or PC and LED will glow. When transmitter rays pass to the receiver, the corresponding receiver is conducting due to that noninverting input voltage is lower than inverting input. Now the comparator output is GND so the output is given to microcontroller or PC. This circuit is mainly used to detect the disturbance in the form of light. Hence addition of suitable filter used to quantify the fluorescence or light intensity emitted by biosensor cells.

Contamination of drinking water with geogenic or anthropogenic arsenic is one of the most severe environmental and public health problems in developing countries, thus calling for reliable, yet simple and cost-effective methods for arsenic monitoring and removal from potable water[3]. Whereas commercially available, colorimetric field test kits have the advantage of being relatively cheap and simple to use for routine arsenic monitoring, they lack the sensitivity and accuracy required to measure arsenic at the toxicologically

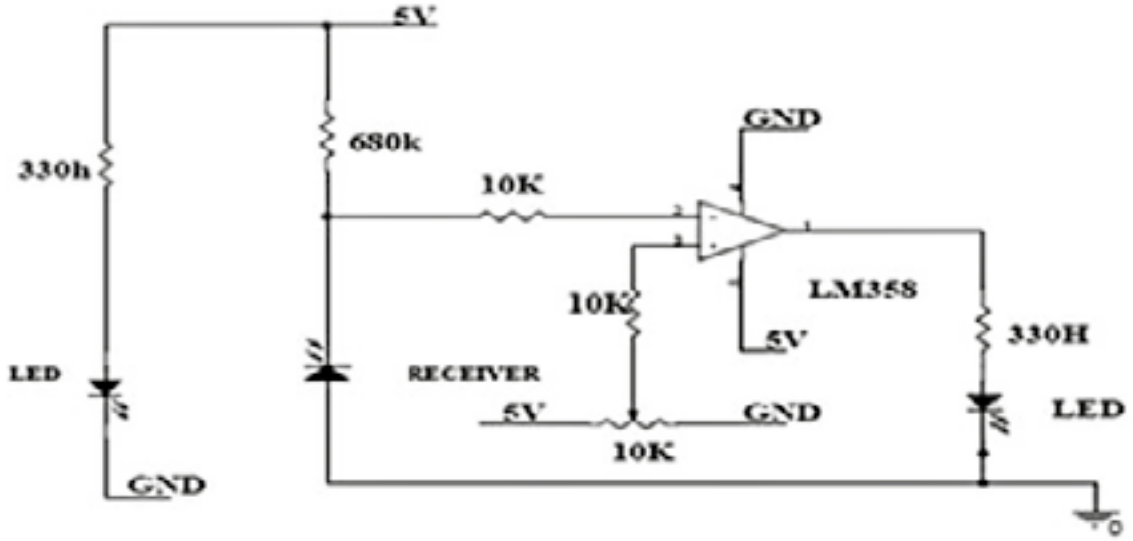


Fig 8 Schematic circuit diagram of fluorometer device

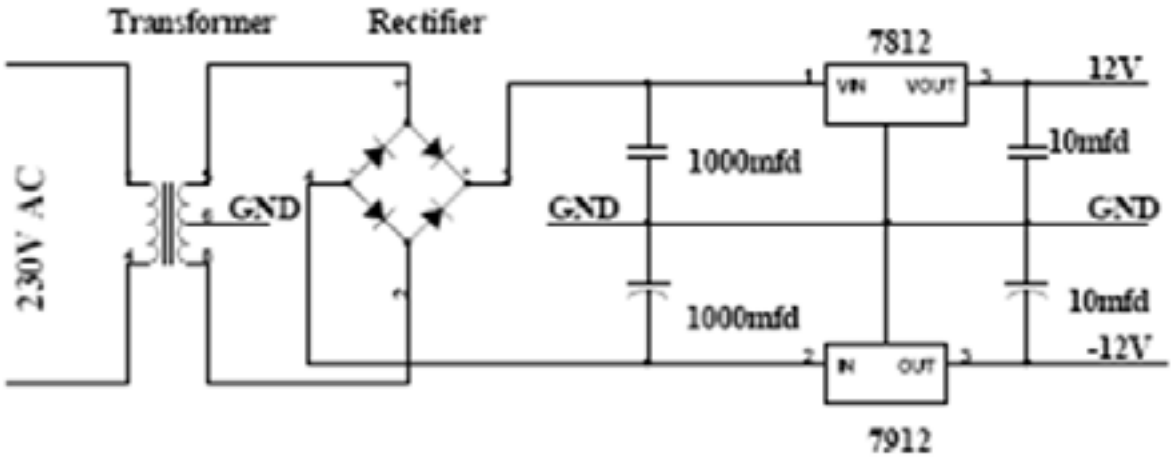


Fig.9 Schematic circuit diagram of fluorometer power supply

relevant concentration range <70 $mgL^{-1}$ frequently found in contaminated potable water sources[10]. Spectroscopic methods of arsenic quantification are reliable and sensitive but do not fulfill the requirement of being affordable for routine monitoring of hundreds of thousands of drinking water wells in the most affected countries Bangladesh and Vietnam[4].

Biosensor measure bioavailable rather than total quantities of chemicals. They can be used

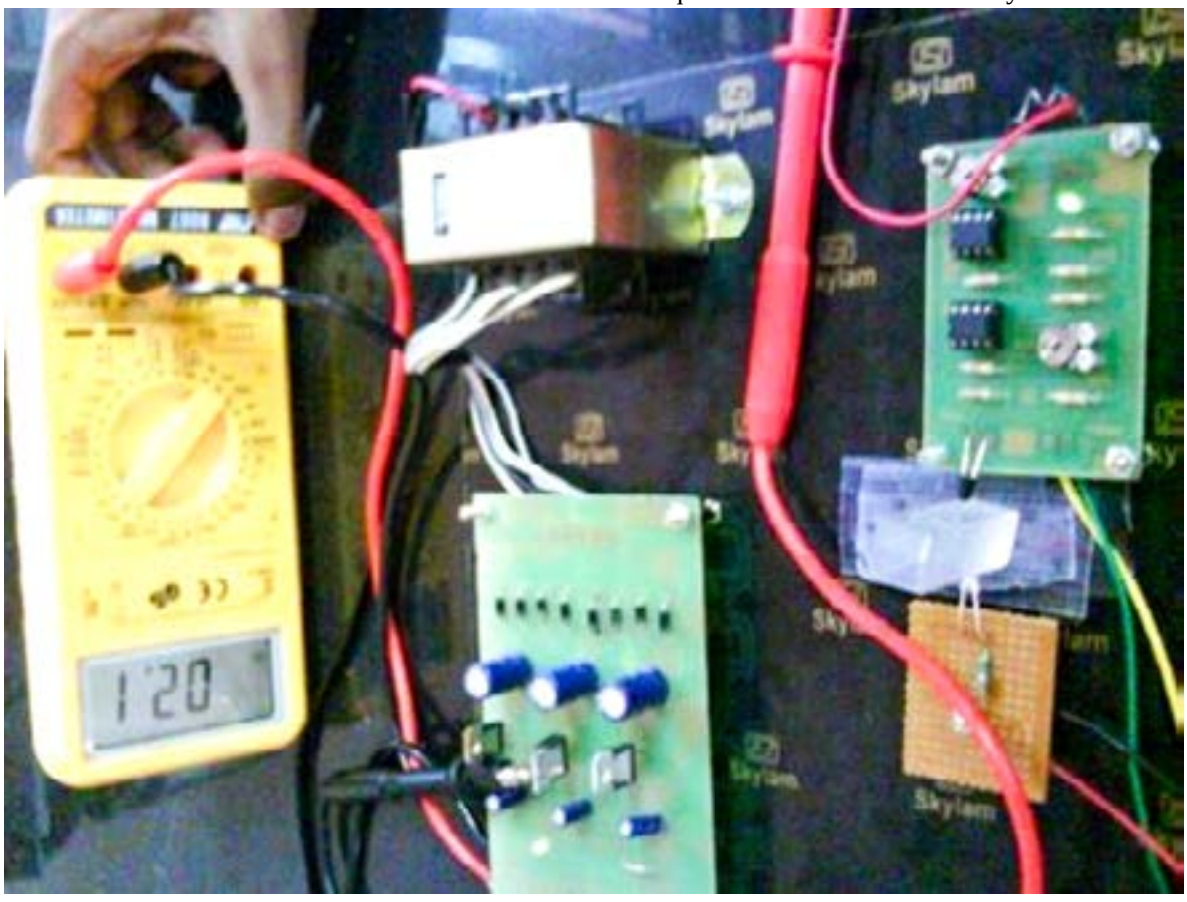


Fig. 10. Developed proto fluorescence sensing system showing deflection when arsenite solution placed in between transmitter and receiver circuit

for spatially restricted environments. Biosensor technology could be a rapid first-line screening for general toxicity or stress. A whole-cell biosensor using the bacteria as the host and a pigment gene/reporter gene as a novel reporter to detect environmental pollutants with high sensitivity[7]. Advantages of bacterial biosensor are Measures bioavailable fraction, inexpensive, produces real-time data, less labor intensive, more sensitive, suitable for field work. Limitations in bacterial biosensor are of short lifetime, lack of genetic stability, limited understanding of applicability to higher organisms, performance dependent on environment of procedure.

Bioreporters have made a brilliant career in research and are generally considered as valuable tools for toxicological and environmental research. Bioreporter organisms have thus little potential as mass products in research. Time also plays a role as well as the success and the public perception of demonstration applications. One promising field technique is a paper-strip test based on genetically engineered *E. coli* bacteria as biosensors. Also, in this case a color reaction indicates the concentration of arsenic in groundwater. The advantage of this technique is the reliable determination of concentrations between 2, 5-75 µg/L. After one-time application the paper-strip can be disposed. The used bacteria are not pathogenic, e.g. one drop of lemon juice makes them harmless. Moreover, this method is simple designed, and the cost of production are kept low (34 Cent a piece), because it is mainly intended for domestic purpose. Simple applications like biosensor field test kits or paper test strips are hampered by the difficulty to compare the resulting color signals with standardized color tables. Therefore, a robust and reliable system for internal calibration would be highly desirable[17].

The high sensitivity of the biosensor cells for arsenite has the additional advantage that unknown samples can be diluted, thereby also diluting possible inhibitory compounds. Qualitative measurements can be performed with the paper strip method, which could facilitate faster identification of samples containing arsenic concentrations substantially above drinking water standards and which subsequently could be analyzed in more detail. Furthermore, the paper strip method could also be used to test the effectiveness of local treatment methods, for instance those targeting the precipitation of arsenic with iron. Despite their potential[6], bacterial whole cell biosensor methods have not received great interest for practical applications until now and have remained mostly research laboratory-based methods. Mainly, this is because the physiological activity and maintenance of the microorganisms can be somewhat difficult to control.

However, almost no reported bacterial biosensors can detect analytes at concentrations below 0.1 µM[9]. Extensive efforts are necessary to increase the sensitivity of bacterial biosensors for widespread use. Arsenite measurements have been performed before with *lacZ* bioreporters under control of the *arsR* promoter using classical spectrophotometrically, chemiluminescence and electrochemical detection[11,12]; or by chromogenic visualization using X-gal[14]. The leaky expression from the *arsR* promoter must be reduced to obtain gradually intensifying blue color formation from X-gal as a function of arsenite concentration. This was accomplished by placing a second *arsR* DNA binding site downstream of *arsR*[14].

The *E. coli* arsenite biosensor cells could be used to produce a color in response to arsenite. For this purpose, β-galactosidase would be a useful reporter protein since many different substrates are available for this enzyme, which will be converted to insoluble colored products. Unfortunately, also the arsenite sensor using *arsR* transcriptionally fused to *lacZ* produced too high background levels of β-galactosidase in the absence of arsenite. Therefore, a second binding site for the *arsR* protein downstream of *arsR* and in front of *lacZ* was introduced. The use of the substrate X-Gal to assess β-galactosidase levels produced intracellularly by the sensor cells. Increasing shades of blue arise at increasing arsenite concentrations to which the cells have been exposed.

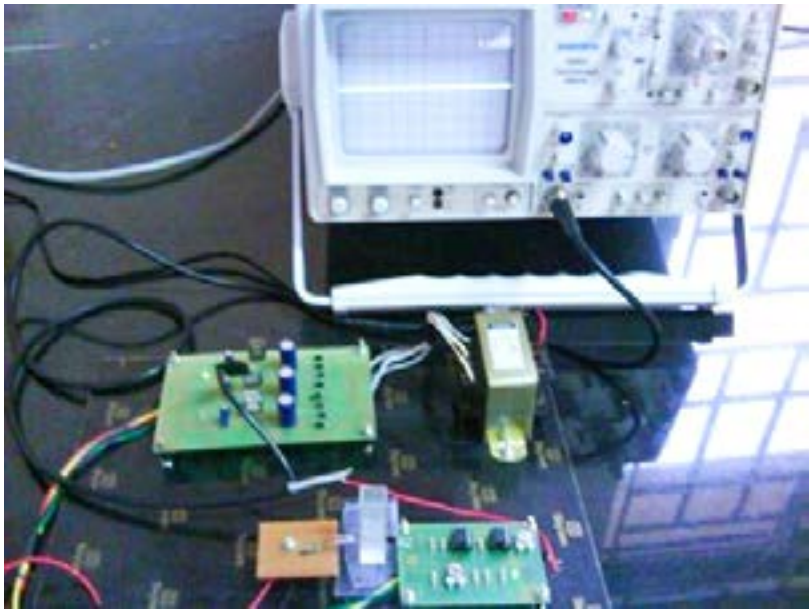

Fig. 11. Developed proto fluorescence sensing system showing deflection when arsenite solution placed in between transmitter and receiver circuit

Although the intensity of the blue color can be measured spectrophotometrically, producing similar calibration curves, the biosensor cells expressing β-galactosidase could also be used on a solid medium for a qualitative strip test. Here to small batches, were dried on a paper matrix. When the paper strips were placed for 30 min in a tube with 1mL of aqueous sample containing arsenite, then taken out and the substrate X-Gal added, blue spots appeared, the intensity of which was proportional to the arsenite concentration. At the amount of biosensor cells used here and after an incubation time of 30 min, the blue color was barely visible at an arsenite concentration around 10µg/L. This was designed in order to create the subjective interpretation of a "negative" test outcome at arsenite levels at or below the current drinking water limits of 10 µg of As/L.

The *arsR* gene controls expression of the reporter gene. In the situation with one *arsR* binding site (black vertical bar), some background expression occurs from the *arsR* promoter (P *ars*), symbolized as a wave line, which leads to the synthesis of *arsR* itself (symbolized as dimer protein) and of the reporter protein. *arsR* binds to its binding site but in the presence of arsenite (AsIII) will lose its affinity for its binding site on the DNA. In this case, repression will be relieved and transcription from the *arsR* promoter will be very high (thick wave line). In case of a secondary *arsR* binding site downstream of *arsR*, only the *arsRm* RNA will be produced as background. *arsR* will bind to both DNA binding sites and prevent RNA polymerase from reading the reporter gene. In the presence of arsenite, *arsR* will again lose its affinity, and expression from the reporter gene will be initiated (Fig.11).

The pUC18 is a high copy number plasmid and thus used to detect very low concentration of arsenite due to cloned *arsR* gene along with its promoter upon arsenite induction. The *gfp* needs no cofactors or substrates for its fluorescence, and exposure to oxygen leads to spontaneous maturation of the fluorophore in the cell. The *gfp* biosensor was developed in order to avoid the use of substrates that are needed for measuring the activities of the luciferase or *β*-galactosidase[14]. The sensitivity of the *gfp* biosensor to arsenite was less than that of the luciferase sensor, with a higher level of detection of 78 ppb. At arsenite concentrations above 7ppb increase of the fluorescence level of individual cells was observed. However, by varying the camera exposure time and induction time, this upper range of detection could be increased to 200 ppb[14]. Subsequent development of our biosensor aims to detect low level of arsenite contamination. The portability of instrument could be improved by acquiring knowledge from physics and electronics basics. The limitation in developing green fluorescence detector lies in the high sensitivity emission filter and optic fiber. These economic factors should be resolved for developing low cost portable sensor which could detect green fluorescence. Currently developed

biosensor could detect and display the values in terms of voltage for varying optical and fluorescence intensity.

The present study has shown that a nonpathogenic biosensor strain of *E. coli* can be controlled and maintained without great problems. These systems could be the basis for either larger scale production of such sensor devices as the paper strips/gadget are for local maintenance and production in quality control laboratories and hospitals, especially in developing countries, where the measurement of arsenic in domestic and drinking water samples is most urgent need of the hour.

## CONCLUSION

Abatement of arsenite becomes unavoidable due to its abundance in nature reported since 1975 in Indian subcontinent. Arsenic has long held as serious public concern which affects biological systems upon ingestion. Arsenic contamination in deeper levels of groundwater due to the use of deep tube wells for water supply in the Ganges Delta. Detection of arsenic levels in potable water can be done with development of whole cell bacterial biosensor through recombinant DNA technology. Arsenite detection through presently available instrumental methods, chemical kits are unreliable. Isolated Pr-ABS-arsR gene was cloned in pBR322/pUC18 and confirmed by agarose gel electrophoresis. Similarly, reporter gene lacZ/gfp was also amplified and cloned in pBR322/pUC18-Pr-ABS-arsR construct. To reduce the background expression second copy of ABS inserted into pBR322/pUC18-Pr-ABS-arsR-lacZ/gfp and pBR322/pUC18-Pr-ABS-arsR-ABS-lacZ/gfp was obtained. The transformed cells with cassette pBR322-Pr-ABS-arsR-ABS-lacZ were selected by replica plating with Luria Bertani agar with ampicillin (100 µg/ml) and in tetracycline (50 µg/ml). The colonies which were not able to grow in tetracycline plate are selected. The selected single colony was used as whole cell bacterial biosensor for the strip preparation. The enzyme β-galactosidase that produces a color reaction in the presence of arsenic. These biosensor cells are also grown in liquid broth, harvested and mixed with ingredients such as sugars, amino acids and gelatin and were pipetted on paper strips and carefully dried at controlled temperature and in partial vacuum. For carryout an assay, thus prepared paper strip is placed in a vial with 1ml of aqueous sample, incubated for 30min at 30°C and taken out. A drop of substrate (X–gal) was added to paper strip the bacterial spot turns to blue color due expression of β-galactosidase. This paper strip may be used as a qualitative measurement of arsenite present in the water samples. In our studies the minimal arsnite detection was observed from 9µg/L. The transformed cells with cassette pUC18-Pr-ABS-arsR-ABS-gfp were selected by blue and white plagues on Luria agar with ampicillin (100µg/ml) and the blue plagues were selected and analyzed for fluorescence emission when exploited in the presence of arsenite. This shows the fluorescence from 7µM of standard arsenite stock solution.

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