DEVELOPMENT OF WHOLE CELL ARSENITE BIOSENSOR AND STUDIES ON PLASMID STABILITY IN REACTORS WITH GENETICALLY ENGINEERED CELL FOR ARSENIC BIOREMEDIATION

A DISSERTATION

Submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF TECHNOLOGY

in

CHEMICAL ENGINEERING

(With Specialization in Industrial Pollution Abatement)

By

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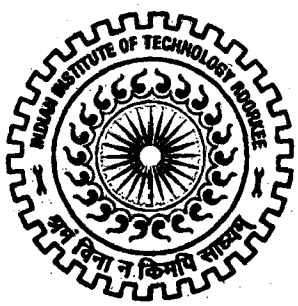

DEPARTMENT OF CHEMICAL ENGINEERING

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in this dissertation entitled **"Development of Whole Cell Arsenite Biosensor and Studies on Plasmid Stability in Reactors with Genetically Engineered Cell For Arsenic Bioremediation"** in partial fulfillment of the requirement for the award of the degree of Master of Technology in the Department of Chemical Engineering with specialization in **"Industrial Pollution Abatement"**, submitted in **Chemical Engineering Department, Indian Institute of Technology, Roorkee, (India)**, is an authentic record of my own work carried out during the period from July 2005 to June 2006 under the guidance of **Dr. C. B. Majumder**, Assistant Professor, Department of Chemical Engineering and **Dr. Partha Roy**, Assistant Professor, Department of Biotechnology, Indian Institute of Technology, Roorkee, (India).

The matter embodied in this dissertation has not been submitted by me for the award of any other degree of this institute or any other institute.

Dated: 29-06-2006
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CERTIFICATE

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

The threat of arsenic pollution to public health and wild life has lead to an increased interest in developing systems that can monitor bioavailable arsenic in potable water. A highly sensitive and selective system for arsenite monitoring was developed from two genetically engineered bacteria, AW10 strain with pMV-arsR-ABS as plasmid named as modified AW10 (mAW10). Plasmid pMV-arsR-ABS provided a reduced background expression in absence of arsenite and its combination with AW10 strain provided high sensitivity at very low concentration of arsenite and to a very wide concentration range covering permissible levels. The bacterial sensing system was found to be highly selective to arsenite over a variety of metal ions and gives dose-response curve linear with coefficient of regression R^2=0.9678 for a long range of arsenite concentration, 0.05 -9 μM. Our work will fetch an important contribution in prevention of diseases caused by arsenic among poor villagers who are exposed to extremely high levels of arsenic.

Also our work focuses on a novel strategy, that a cell can be forced to maintain plasmid, if the plasmid is encoded with genes necessary for its survival in that environment. The cells in arsenite environment and lacking chromosomal *ars* operon, required for self defense against toxic environment of arsenic are forced to maintain plasmid which have *arsR* gene encoded on them. As arsenite is bioaccumulated by arsR proteins of the cell, this works as new defense mechanism to some extent in the cell. Cells induced by arsenite at late exponential phase were found to maintain high plasmid stability in bioreactors. The problem of plasmid stability can be over come by exploitation of recombinant organisms for the large-scale, commercial production of foreign proteins for metal bioaccumulation under arsenic environmental pressure.

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CHAPTER 1

INTRODUCTION

1.1 ARSENIC IN ENVIRONMENT

Life arose in an alien and hostile environment of this earth. Before the atmosphere became oxidizing, the concentrations of dissolved metal ions in primordial oceans were undoubtedly much higher than today. One of the earliest challenges of the first cells would have been the ability to detoxify heavy metals, transition metals and metalloids, including As(III). The presence of arsenic resistance (*ars*) genes in the genome of every living organism sequenced to date illustrates that *ars* genes must be ancient [1]. The minimum set of genes needed for arsenic resistance is *arsRBC* [3]. The ubiquity of environmental arsenic provides the selective pressure that maintains resistance genes in present day organisms from *E.coli* to humans [1].

Arsenic is the 20th most abundant element in the earth's crust, and found ubiquitously in nature. About 155,000 metric tones of arsenic are released into the environment each year [2]. Arsenic enters the biosphere primarily by leaching from the geological formations. Anthropomorphic sources include mining and arsenical – containing fungicides, pesticides and herbicides [1]. Current estimates are that 35-50 million people in the West Bengal and Bangladesh area, over 10 million in Vietnam, and over 2 million in China are exposed to unacceptable arsenic intake through potable water consumption [4]. The safety limit for arsenic in drinking water for most European countries and United states, 10 μg/L; elsewhere, 50 μg/L [4].

Thus, this problem of arsenic pollution influenced us to choose our objective of work as easy determination of arsenic in potable water and to work for bioremediation of arsenic polluted water by recombinant cells. **Our objective is**

- Development of highly sensitive and selective whole cell arsenite biosensor for measurement of bioavailable arsenite in contaminated water.
- To optimize the developed biosensor for various arsenite concentrations.

- To carry out test for Selectivity of the developed biosensor.
- To study plasmid stability of a recombinant bacteria at various arsenite concentrations.
- To optimize the reactor conditions to maximize the plasmid stability of the recombinant cell population for maximizing the production of arsenic bioaccumulating ArsR protein.

Systemic and chronic exposure to arsenic is known to lead to serious disorders, such as vascular diseases (Blackfoot disease and hypertension) and irritations of the skin and mucous membranes as well as dermatitis, keratosis, and melanosis. Inorganic arsenic is a human carcinogen, and ingestion of inorganic arsenic increases the risk of developing cancer of the bladder, liver, kidney, and skin. The clinical manifestations of arsenic intoxication are referred to as arsenicosis. Currently, the largest case of arsenic poisoning takes place in Bangladesh. It is estimated that out of 4 million tubewells, 1.12 million are affected by arsenic contamination and that between 20 and 30 million people (15-25% of the population of the Bangladesh) are exposed to arsenic levels above 50μg/L.

Arsenic is a toxic metalloid which belongs to group XV of the periodic system together with nitrogen, phosphorous, antimony and bismuth and it has two biologically important states, As(V) and As(III), as the oxyacids arsenic acid (H_3AsO_4) or arsenous acid, also called arsenic trioxide (As_2O_3)[1,3]. In solution at neutral pH, arsenic acid exists as the arsenate oxyanion. The pK_a of arsenous acid is 9.2, so that, at neutral pH, it would be primarily present in solution as neutral $As(OH)_3$. As(III) as a soft metal ion, forms strong bonds with functional groups such as thiolates of cysteine residues and the imidazolium nitrogens of histidine residues[1]. Arsenite is uncharged at neutral pH and appears to gain access to the cytoplasm by less specific mechanisms, possibly including diffusion across the membrane. It crosslinks sulfhydryl (thiol) groups of enzymes, forming stable adducts that permanently disable the enzyme. This mechanism is even more destructive to the cell than that of arsenate [5]. Arsenite has a high affinity for thiol groups and affects respiration by binding to the vicinal thiols in pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. It also affects the function of the glucocorticoid receptor [6].

Arsenate is a chemical analog of phosphate and can uncouple mitochondrial oxidative phosphorylation. Arsenate enters the microbial cells readily through phosphate uptake proteins. Its primary mode of toxicity is then to displace phosphate in the production of adenosine triphosphate (ATP), the primary energy currency of the cell. The resulting molecules hydrolyze spontaneously, causing the cell to deplete its energy sources rapidly [5].

Organisms take up As (V) via phosphate transporters and As(III) by aquaglyceroporins. As(V) is reduced to As(III) which is either extruded from the cells or sequestered in intracellular compartments, either as free arsenite or as conjugates with GSH or other thiols[1]. Arsenic is an essential toxin, it is required in trace amounts for growth and metabolism but is toxic at elevated concentrations. Arsenic is used as an osmolite in some marine organisms and its use for energy is widespread in prokaryotes with representative organisms from Crenarchaeota, thermophilic bacteria, low and high G + C gram-positive bacteria, and Proteobacteria [5].

In higher eukaryotes, glutathione reduces arsenate to arsenite, which then accepts a methyl group from S-adenosyl-methionine producing MMA or DMA with the help of methyltransferase. The methylated forms of arsenic, such as monomethylarsonic (MMA) and dimethylarsonic acid (DMA) are less toxic and are main route of detoxification in some mammals [6].

1.2 Toxicity of Arsenic compounds

Arsenic occurs in four oxidation states: As^{+5}, As^{+3}, As^{0} and As^{-3}.

Arsenate

This oxyanions is analog of phosphate, and as such it is potent inhibitor of oxidative phosphorylation, the key reaction of energy metabolism in metazoans, including humans.

Arsenite

It is the most toxic of arsenic oxyanions. It readily binds to reactive sulphur atoms (SH groups) of many enzymes, including those involved in respiration.

Arsenic trioxide(As_2O_3)

It is the most common form of arsenic used for a variety of agricultural, manufacturing and medical purposes. It is highly toxic.

Methylated forms of arsenate and arsenite

Compounds such as methylarsonic acid (MMA^V), monomethylarsonous acid (MMA^{III})and dimethylarsenic acid (DMA^V) are produced by algae and as excretory products of animals.

Arsines

Arsines in the -3 oxidation state, occurring as highly toxic gases, such as H_3As and $(CH_3)_3As$.

Organoarsenic compounds

Arsenobetaine is molecular analog of osmotic regulating compound, betaine, where arsenic substitutes for the original nitrogen atom. They occur in marine animals but are not toxic to animals that eat these organisms, including humans.

1.2 BIOSENSOR

A biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element. Biosensor can be clearly distinguished from a bioanalytical system which requires additional processing steps, such as reagent addition. Furthermore a biosensor can be distinguished from a bioprobe which is either disposable after one measurement, i.e. single use, or unable to continuously monitor the analyte concentration.

The *biological recognition element* of a biosensor interacts selectively with the target analyte(s), assuring the selectivity of the sensors. These elements can be classified into two main classes: biocatalysts (enzymes, microorganisms, tissue materials) and bioligands (antibodies, nucleic acids, lectins). Their application is limited mainly by the

existence of efficient preparation methods (e.g. separation, purification, viability of microorganisms or living cells, etc.).

The selectivity of the biosensor for the target analyte is mainly determined by the biorecognition element, whilst the sensitivity of the biosensor is greatly influenced by the transducer.

A proper functioning of the biosensors requires the presence of an intermediary compound, a so-called mediator, which shuttles redox equivalents between the recognition element and transducer. This supplementary step in the biosensing chain usually results in the increase of the biosensor's selectivity and sensitivity.

1.2.1 APPLICATONS OF BIOSENSORS

Biosensor technology can enable investigators to detect extremely small amounts of chemical or biochemical agents in a biological medium. Resolution of parts per billion can be obtained and concentrations ranging from 500 to 500,000 cells/mL.

1.2.1.1 Health

Biosensors can effectively be used to monitor the glucose levels in diabetic patients. These monitoring systems can ease the hassle of controlling diabetes. The glucose monitoring research has been focusing on in vivo applications. Scientists are pursuing noninvasive systems for use at home by diabetics based on the direct measurement of the reflection and transmission spectra in the Near Infrared (NIR) Spectrum. Detection can be done noninvasively because skin tissue is quite transparent to the NIR. Presently, glucose needle enzyme electrodes are being successfully tested on lab rats by implanting the sensor and the microflow cannula right below the skin. Researchers are working to modify the sensitivity of the *in vivo* glucose sensors to be more comparable to the in vitro sensors.

Biosensor technology is also being put to use by the United States military. The US Navy has created underwear woven from conductive polymers that can provide data on the wearer's injuries. The sensors can recognize when a soldier is bleeding and

whether the cut is in a vein or artery, depending on the oxygen content in the blood. The US Army built a pill-sized computer that soldiers can swallow. The computer tracks the health of the soldiers and may detect stress levels.

1.2.1.2 Drug discovery

Conventionally, drugs are discovered through a time consuming, tedious process. Biosensors have tremendous potential in speeding up this process. Biosensors can detect the interaction between a particular target and a possible drug without using markers or the detection of color changes or fluorescence. Biosensors can quickly measure how well a potential drug binds to a target and, by eliminating markers, biosensors eliminate potential causes of interference. Test samples do not need to be purified and can be reused.

1.2.1.3 Pathogen Detection in Food

As the occurrence of food-borne illness rises, governments are passing stringent regulations as to tolerable amounts of contamination in foodstuffs. However, conventional methods of pathogen detection require time consuming steps to arrive at a useable measurement. This process, usually taking 7 to 10 days, is much too long for perishable food, which usually enters and leaves the marketplace within 2 to 3 days. Biosensor technology can significantly reduce this time as well as detect even smaller amounts of pathogens with fewer false positives.

1.2.1.4 Environment Monitoring

Environmental pollution has an impact on human health and can therefore be considered being connected to life quality. Depending on the purpose, sensitive and selective methods are needed for both quantitative and qualitative determination of target analytes. Numerous such techniques are already available. However, the requirements for low cost, short time of analysis, sensitivity, simplicity, selectivity, stability and reliability can still not be met by a single analytical method. Biorecognition-based techniques are potential

candidates to fulfill many of the above requirements, due to the general high selectivity, sensitivity and speed of interaction between the biological component and the analyte itself.

Stabilised heavy metal ions (e.g. via forming metal complexes) are not available to the biorecognition element, and therefore, are considered harmless to living beings as long as they are in their bound forms. Essential metals are catalysts in biochemical reactions, function as stabilisers of protein structures and bacterial cell walls, and serve in maintaining osmotic balance.[7]

Several metal-binding proteins were used as biorecognition elements, namely a prokaryotic metallothionein (GST-SmtA), mercury-responsive proteins (MerR and MerP), and a synthetic phytochelatin (EC20). The principle of the capacitance measurement used as signal transduction has been described earlier in details. Briefly, the mentioned proteins selectively bind heavy metal ions via thiolate complex formation, and could be immobilized on the surface of a suitable transducer, e.g. a gold electrode [8]. Conformational changes within the immobilized protein layer are occurring when the metal ions are binding to the protein. The transducer translates these conformational alterations into a measurable change of capacitance.

Sensing Contamination

When the bacteria come into contact with substances with toxic biological effects, the amount of light given out is reduced. This makes them ideal for assessing the toxicity of industrial effluent or soil.

The use of biosensors allowed assessment of the site in weeks when previously it had been taking months [9]. Also, the biosensor survey discovered an area of toxicity that had been overlooked by previous surveys.

1.3 PLASMIDS

The use of plasmids as vectors is almost indispensable in genetic engineering for the expression of desired foreign genes in cells. Plasmids are non-essential

extrachromosomal DNA elements. They replicate autonomously and are inherited with different rates of fidelity, but are not essential for cell viability.

Desirable characteristics for useful vectors include

1. High copy number
2. Possession of several unique restriction endonuclease cleavage sites,
3. Small size
4. Genetic stability
5. Screening markers (e.g. antibiotic resistance gene encoded by the plasmid),
6. Simple procedures for transfer into host

A small plasmid imposes less of a metabolic burden on the host and also facilitates transformation. The most important parameter is that the expression plasmid should be stably maintained in the host for several generations.

1.3.1 Plasmid Stability

The productivity of a bioreactor employing recombinant strains is largely affected by the degree to which the plasmid-free (P^-) cells are generated and propagated and is responsible for further complications in scale-up which is required for commercialization.[10-13].

The P^- cells are generated from plasmid-harboring (P^+) cells by segregational instability which is caused by defective partitioning of the plasmids between the daughter cells during cell division [14].

Structural instability originates from changes in the plasmid itself such as point mutation, deletion, insertion and rearrangement in the plasmid DNA [15,16]. The resulting cells are non-productive.

The P^+ cells usually grow more slowly than the P^- cells because the P^+ cells have to synthesize more DNA, mRNA and protein [17]. This leads to a lower maximum growth rate (μ_{max}) for P^+ cells [11,17,18]. In addition, the growth rate of P^+ cells also depends on toxicity of the coded proteins and strength of promoters. Thus, once generated in the reactor, P^- cells may propagate rapidly, leading to a mixed population

with the P^- proportion of population increasing with each generation, leading, in turn, to poor economics of the total bioprocess[10,11,13].

Although continuous systems are highly productive for many microbial production processes, their application is more limited for recombinant organisms because of plasmid instability. The situation is more complicated in continuous processes where the cells go through a greater number of generations than in batch process.

1.3.1.1 Strategies for Improving plasmid stability

Selective methods

1. The selective methods include maintaining selection for antibiotic resistance by use of antibiotics in the growth medium, complementation of host auxotrophy markers on plasmid vectors.
2. Lysogenic phage repression and incorporation of suicide proteins and RNA whose synthesis is repressed in the presence of the plasmid.

Non-selective methods

Non-selective methods include incorporation of partition loci to obtain controlled partitioning of the plasmid to the daughter cells during cell division [19].

Environmental pressure helps in plasmid maintenance for survival of cell

Our work focuses on another strategy, that a cell can be forced to maintain plasmid, if the plasmid is encoded with genes necessary for its survival in that environment. The cells in arsenite environment and lacking chromosomal *ars* operon, required for self defense against toxic environment of arsenic are forced to maintain plasmid which have *arsR* gene encoded on them. As arsenite is bioaccumulated by ArsR proteins of the cell, this works as new defense mechanism to some extent in the cell.

Introduction of inducer to cells for transcription of plasmids along with inoculation in fresh media reduced stability suddenly to a great extent initially but later stability improved with passage of time. Where when induction of plasmid was done in late log phase, plasmids kept high stability due to external environmental pressure of toxic arsenite, forcing the cell to maintain the production of plasmid DNA for its resistance to arsenite.

1.3.2 Segregational Loss

Segregational loss occurs when a cell divides such that one of the daughter cells receives no plasmids. High copy number plasmids are usually distributed randomly among daughter cells following a binomial distribution. The segregational loss of plasmid is influenced by many factors, such as dissolved oxygen, temperature, medium composition, and dilution rate in a chemostat (Fig. 4.11).

Segregational instability arises from a failure to distribute plasmids to both daughters at cell division and is affected by multiple factors including host and plasmid genotype, medium composition and growth rate. Although plasmid load has no effect on the rate at which plasmid-free cells arise, it can cause a dramatic increase in the rate at which they accumulate in cultured cells. After an initial period of slow accumulation, plasmid-free cells take over the culture. Plasmid- free cells can be controlled by using selectable markers in the plasmid. Antibiotic resistance selection is the most common form of selectable markers. However, in bioremediation applications the stability of recombinant plasmid in the absence of selection is of considerable importance [20].

1.4 CONTINUOUS IMMOBILIZED REACTOR

The ability of maintaining high cell concentration in the reactor at high dilution rates provides immobilized cell reactors a number of advantages over chemostats.

- More cell means that the reactor contains more catalyst and therefore high conversion rates can be achieved.
- Immobilized cell reactors are also more stable than chemostats. In a chemostat, a temporary (transient) increase in the dilution rate will cause a rapid drop in cell numbers. The entry of a slug of toxic substances in the feed will have the same effect. It will take time for the cells numbers to build up again. Since the cells are not as easily washed out of an immobilized cell reactor, the recovery time will be quicker and fall in biomass concentration will be smaller.

Recombinant mAW10 strain was immobilized with granular activated carbon (GAC) as supporter matrix for production of ArsR protein. Immobilization could also decrease the probability for segregational plasmid loss and overgrowth of plasmid-free cells. It was found that Cell density, plasmid stability and ArsR protein productivity were higher than without immobilization.

CHAPTER 2

LITERATURE REVIEW

2.1 Arsenic Resistance Mechanism

2.1.1 Arsenic Resistance mechanism in *Escherichia coli.*

Arsenic uptake in *E.coli*: GlpF is an aquaglyceroporin, a member of the aquaporin superfamily. Aquaglyceroporins are multifunctional channels that transport neutral organic solutes such as glycerol and urea. Chemical properties of Sb (III) and As (III) are very close and GlpF plays its role in their transport into the cell. Uptake of As (V) is via phosphate transport system. Under abundant phosphate conditions, the high V_{max} but less specific Pit system fulfills the phosphate need of the cell and leads also to arsenate accumulation. Under conditions of phosphate starvation, the more specific Pst-system is induced. Pst discriminates between phosphate and arsenate 100-fold better than Pit. Thus one way for the cell to adapt to arsenate stress is to inactivate the Pit system by mutation, which leads to moderate arsenate tolerance due to the discrimination between arsenate and phosphate by the Pst system (Fig. 2.1) [21].

Arsenate is reduced to arsenite by the bacterial ArsC enzyme and glutathione and glutaredoxin serves as the source of reducing potential. The bacterial detoxification of arsenic is often based on inducible ion efflux systems that reduce the intracellular concentration of arsenic by active export. Since anion export from the bacterial cells is driven by the chemiosmotic gradient, simple arsenic As (III) efflux systems are composed of just on efflux protein. As (V) cannot, however be transported with this system [23]. The solution to the problem of As (V) efflux is the enzyme arsenate reductase (ArsC in case of *E.coli*), which catalyzes the reduction of As (V) to As (III), the substrate of the efflux system. Thus this enzyme extends the spectrum of resistance to include both As (III) and As(V)[24].

The resistance of certain bacteria to antimonite and arsenite is conferred by the ars operon. The ars operon consists of five genes: three structural genes (arsA, arsB, and arsC) and two regulatory genes (arsR and arsD). The structural genes form an efflux

visible as blue-green light at 490nm. The fatty acids are recycled back into the aldehyde substrate by a multienzyme fatty acid complex consisting of three proteins, a reductase, transferase, and synthetase encoded by the *lux*C, D, and E genes, respectively. *lux* fusions can consist of just the *luxAB* complex or the complete *luxCDABE* cassette. If only the *luxAB* genes are used, the cells must be supplied with an aldehyde substrate, typically decanal, before light production is possible. Utilization of the entire *luxCDABE* cassette, however, allows for a completely self contained bioluminescent response, negating the requirement for any exogenous substrate additions. Therefore, the presence of a signal indicates that the bioreporter has sensed a particular target agent (Arsenite) in the environment. Table 2a and Table 2b lists some of the arsenic biosensors developed.

Table 2a. Developed Arsenic Biosensors

Study	New Plasmid	Arsenic sensing gene	Reporter gene	Host strain	Detectable metals	Detection limits
Ji, G. and S Silver. 1992. [31]	----------	*arsR,arsB,arsC* of *Staphlocuoccus aureus* plasmid pI258	Tested *ars* operon in various hosts.	*S.aureus, Bacillus subtilis* *Escherichia coli*	Arsenate Arsenite Arsenate Arsenite	About 1μm higher than Taurianinen et al., 1997
Ramanathan, et al., 1997, [30]	pRLUX	*arsR, arsD*	*luxAB*	*E.coli* (JM109)	Antimonite Arsenite	10^{-15}M
Scott et al., 1997 [28]	pBGD23	*arsR, arsD*	*lacZ*	*E.coli* (JM109)	Antimonite Arsenite	10^{-7}M
Corbisier et al., 1993. [8]	pC200	*arsB* of *S.aureus* plasmid pI258	*luxAB* genes from *Vibrio harveyi*	*E.coli* (HB101) *S.aureus*(RN4220)	Arsenite Arsenate Arsenite	About 1μm higher than Taurianinen et al., 1997
Taurianinen et al., 1997. [7]	pT0021	*arsR* and promoter of *S.aureus* plasmid pI258	*lucFF* from *Photinus pyralis*	*S.*aureus(RN4220) *B.subtilis*(BR151)	Arsenite Arsenite Arsenate	100 nm 3.3 μm 330 μm
Roberto et al., 2002. [32]	pIRC140	Promoter, *arsR, & arsD*	GFP of *Aequorea victoria*	*E.coli*	Arsenite Arsenate	μm to nm, 1 ppb

pump capable of extruding arsenite and antimonite out of the cell. ArsA is an ATPase subunit that provides the energy necessary to drive the protein pump by ATP hydrolysis. ArsB is the membrane transport that forms the ion-channel through which antimonite and arsenite are extruded out of the cell. The third structural gene arsC code for the arsenate reductase that converts arsenate (AsV) to arsenite (AsIII), which can in turn be detected by the efflux pump. The expression of the operon genes is controlled by the regulatory proteins. ArsR controls the basal expression of the operon, while ArsD controls the maximal expression. In the absence of antimonite/arsenite, the ArsR binds to the operator/promoter (O/P) region of the ars operon preventing transcription of the genes. When the toxic metalloids are present, they bind to ArsR producing a conformational change that leads to its dissociation from the O/P region, allowing for transcription and further expression of the operon genes (Fig. 4.2).

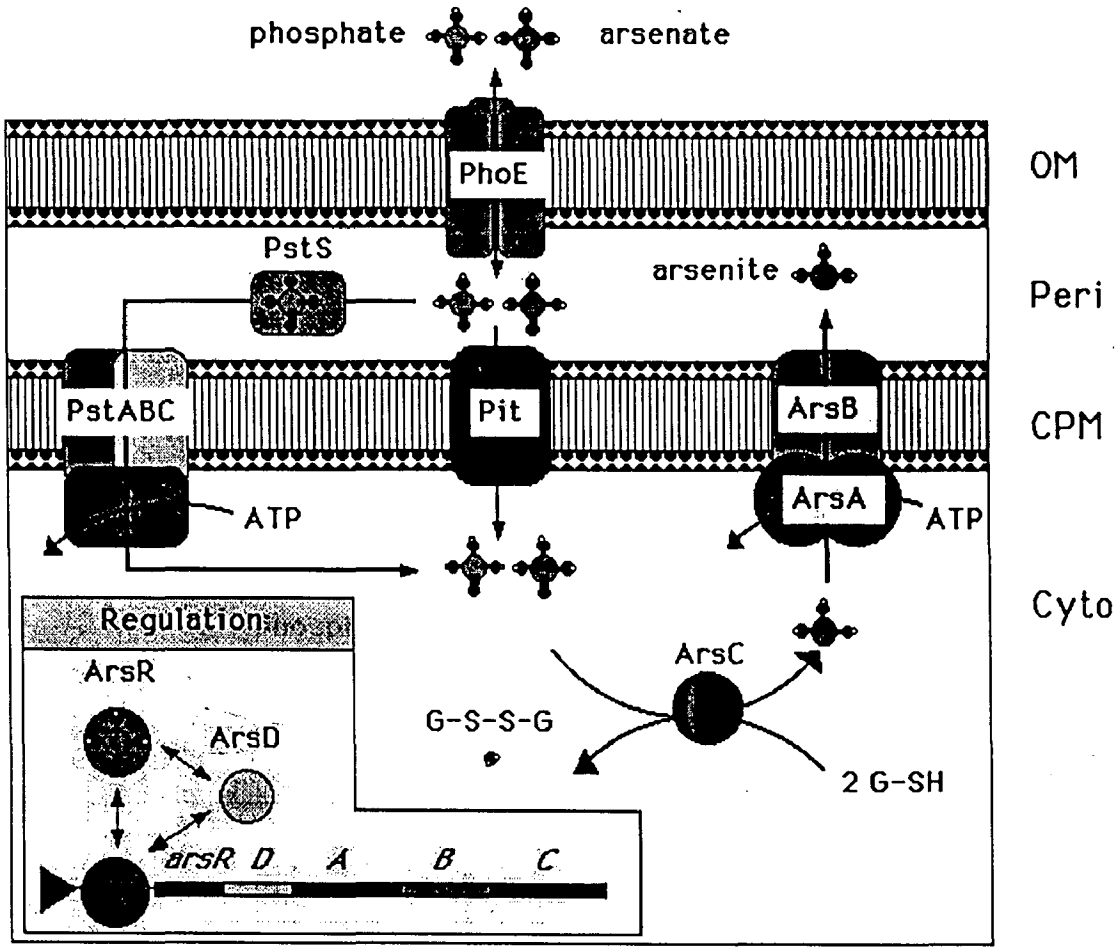


Fig. 2.1: Transport and resistance to arsenate in *E. coli*. Arsenate and phosphate enter the periplasmic space through the outer membrane porin, the PhoE protein. Both anions are transported into the cytoplasm by the Pit protein or the Pst system (which is more specific for phosphate). Within the cell, arsenate is reduced to arsenite by the ArsC protein (dependent on glutaredoxin and glutathione) and arsenite is pumped out of the cell by the ArsAB efflux ATPase. The arsRDABC operon is regulated by the ArsR repressor protein and the ArsD co-regulator protein. OM, outer membrane; Peri, periplasmic space; CPM, cytoplasmic membrane; Cyto, cytoplasmic space; G-SH, reduced glutathione; G-S-S-G, oxidized glutathione. Phosphate, arsenate and arsenite are shown as tetragonal or trigonal oxyanions[21].

2.1.2 Exploitation of Bacterial Defense Mechanism against Arsenic

Bioluminescent Bioreporters are bacteria that can be genetically engineered to achieve bioluminescence in response to specific contaminants (arsenic here). They contain two essential genetic elements: a promoter gene and a reporter gene. With reporter gene fusion technology a promoterless reporter gene that lacks regulatory signals

of its own, is coupled to the regulatory signals of *ars* promoter. The promoter gene (*ars* promoter) is turned on (transcribed) when the contaminant (arsenite), is present in the cell's environment. In a normal cell, the promoter gene is linked to other genes that are likewise transcribed and then translated into proteins that help the cell in either combating or adapting to the contaminant e.g., *ars* operon containing *arsA, arsB, arsC, arsD,* and *arsR* is present in *E.coli* to provide resistance to arsenic oxyanions. ArsR is an arsenite sensing protein. We take advantage of the biochemical capacities of ArsR. It has two binding capacities: In the absence of arsenite, it binds to a specific element on the DNA and thereby prevents the arsenic defense genes from becoming transcribed by RNA polymerase. Repression, however is not complete and small amounts of ArsR, the arsenate reductase and the arsenite pump are always present. When arsenite enters the cell, ArsR changes its habits; it will immediately bind to the arsenic compound and lose affinity for the DNA binding site with the result that the protein "falls off" the DNA. As a consequence, ArsR no longer represses the defense mechanism so that the arsenic pump, and the arsenate reductase are produced by the cell in larger amounts[25, 26, 27].

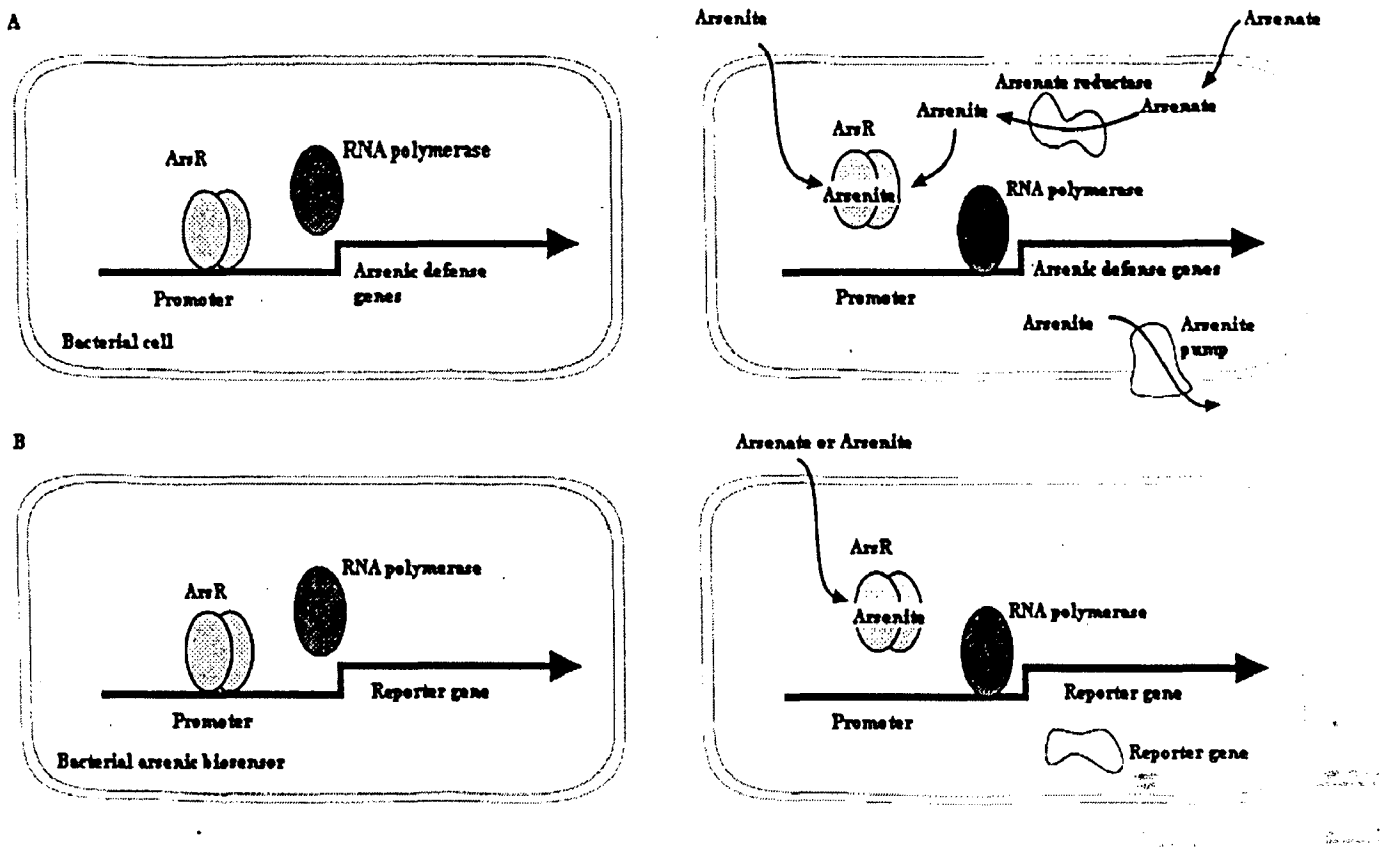


Fig. 2.2: Principle of the arsenic biosensors. A) The ArsR regulatory protein binds to a specific DNA element and thus inhibits the expression of the genes coding for the arsenic defense mechanism. When arsenite enters the cell, it binds to ArsR. ArsR dissociates from the DNA and RNA polymerase can access. The arsenic defense genes (the ArsR protein itself, the arsenite pump and the arsenite reductase) are expressed in high amounts. B) In the biosensor cells, the ArsR control DNA element is coupled to a reporter gene. Expression of the reporter gene is inhibited by the ArsR, but when arsenite enters the cell, it again dissociates and the reporter gene product is synthesized. These products are either luciferase, green fluorescent protein or β-galactosidase[27].

In case of a bioreporter, these genes, or portions of the genes, have been removed and replaced with a reporter gene. Consequently, turning on the promoter gene now causes the reporter gene to be turned on. Activation of reporter genes (*lux* gene cassette) leads to production of reporter proteins that ultimately develops some time of detectable signal (light). Many of the bioluminescence assays utilize the bacterial *lux* system, consisting of five genes *luxC,D,A,B,* and *E. luxA* and *B* together encode for the enzyme luciferase which is responsible for generating the bioluminescent response. Luciferase converts long-chain aldehydes into fatty acids with a concomitant production of photons,

visible as blue-green light at 490nm. The fatty acids are recycled back into the aldehyde substrate by a multienzyme fatty acid complex consisting of three proteins, a reductase, transferase, and synthetase encoded by the *lux*C, D, and E genes, respectively. *lux* fusions can consist of just the *luxAB* complex or the complete *luxCDABE* cassette. If only the *luxAB* genes are used, the cells must be supplied with an aldehyde substrate, typically decanal, before light production is possible. Utilization of the entire *luxCDABE* cassette, however, allows for a completely self contained bioluminescent response, negating the requirement for any exogenous substrate additions. Therefore, the presence of a signal indicates that the bioreporter has sensed a particular target agent (Arsenite) in the environment. Table 2a and Table 2b lists some of the arsenic biosensors developed.

Table 2a. Developed Arsenic Biosensors

Study	New Plasmid	Arsenic sensing gene	Reporter gene	Host strain	Detectable metals	Detection limits
Ji, G. and S Silver. 1992. [31]	----------	*arsR,arsB,arsC* of *Staphlocuoccus aureus* plasmid pI258	Tested *ars* operon in various hosts.	*S.aureus, Bacillus subtilis* *Escherichia coli*	Arsenate Arsenite Arsenate Arsenite	About 1μm higher than Taurianinen et al., 1997
Ramanathan, et al., 1997, [30]	pRLUX	*arsR, arsD*	*luxAB*	*E.coli* (JM109)	Antimonite Arsenite	10^{-15}M
Scott et al., 1997 [28]	pBGD23	*arsR, arsD*	*lacZ*	*E.coli* (JM109)	Antimonite Arsenite	10^{-7}M
Corbisier et al., 1993. [8]	pC200	*arsB* of *S.aureus* plasmid pI258	*luxAB* genes from *Vibrio harveyi*	*E.coli* (HB101) *S.aureus*(RN4220)	Arsenite Arsenate Arsenite	About 1μm higher than Taurianinen et al., 1997
Taurianinen et al., 1997. [7]	pT0021	*arsR* and promoter of *S.aureus* plasmid pI258	*lucFF* from *Photinus pyralis*	*S.*aureus(RN4220) *B.subtilis*(BR151)	Arsenite Arsenite Arsenate	100 nm 3.3 μm 330 μm
Roberto et al., 2002. [32]	pIRC140	Promoter, *arsR, & arsD*	GFP of *Aequorea victoria*	*E.coli*	Arsenite Arsenate	μm to nm, 1 ppb

Table 2b. Developed Arsenic Biosensors

Study	New Plasmid	Arsenic sensing gene	Reporter gene	Host strain	Detectable metals	Detection limits
Taurianinen et al., 1999. [29]	pT0031	Promoter and *arsR* of *E.coli* plasmid R773	*lucFF* from *Photinus pyralis*	*E.coli*(MC1061) *E.coli*(AW3110)	Arsenite Arsenate Arsenite Arsenate	33 nm 33 µm Similar to MC1061
Petänen et al., 2001. [33]	pTPT21 pTPT31	Promoter and *arsR* of pI258 Promoter and *arsR* of R773	*lucGR* of *Pyrophorus plagiophthalamus* *lucGR*	*E.coli*DH5α, *Pseudomonas fluorescens* OS8 *E.coli*DH5α, *P.fluorescens* OS8	Arsenite Arsenate Arsenite Arsenate	----------
Alexander et al., 2000. [34]	----	*arsB* as Promoter *arsB* as Promoter	*lacZ* *luxAB*	*E.coli*	Arsenite Arsenate Chromated copper arsenate	5 µg As/L 100 µg As/L 10 µg As/L
Stocker et al., 2003. [35]	pMV-arsR pMV-arsR-ABS pJAMA-arsR pJAMA-arsR-ABS pPR-arsR pPR-arsR-ABS	*arsR* *arsR* and *arsR* DNA binding site. *arsR* *arsR* and *arsR* DNA binding site. *arsR* *arsR* and *arsR* DNA binding site	*lacZ* *lacZ* *luxAB* *luxAB* *gfp* *gfp*	*E.coli*DH5α *E.coli*DH5α *E.coli*DH5α	Arsenite Arsenite Arsenite Arsenite Arsenite Arsenite	 8-80 µg As/L

2.2 Whole-cell Biosensors

The development of whole-cell biosensors involves coupling the genes of proteins capable of molecular recognition with reporter genes, such as luciferase, β-galactosidase and GFP. The sensing systems developed are based on genetically designed bacteria that are engineered to generate a signal when the target analyte is present [28]. These sensing systems employ bacteria harboring plasmids with reporter genes. An analytical signal can be produced within the cell by inducing the reporter gene in the presence of the target analyte (Fig. 2.3).

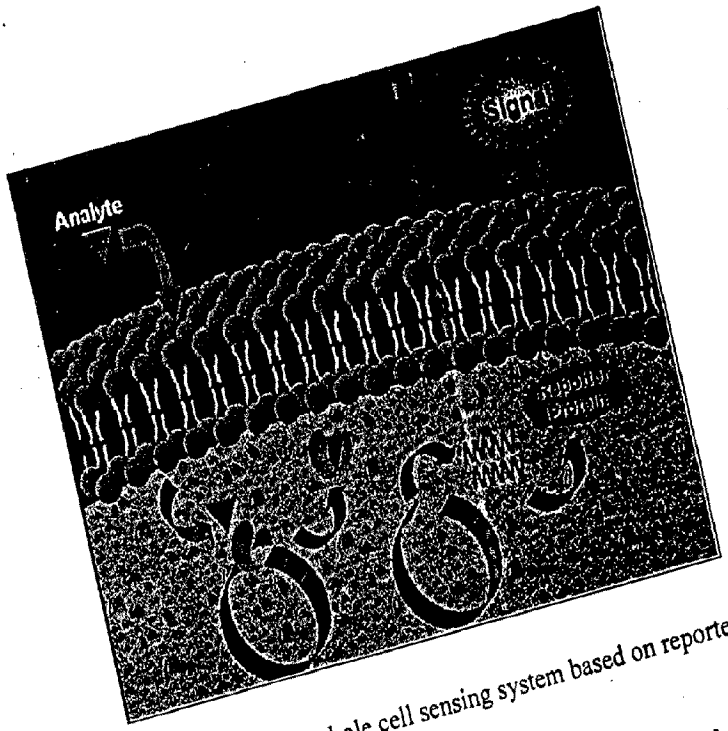


Fig. 2.3: Schematic depicting a whole cell sensing system based on reporter genes

Biosensors based on whole cells are used for a variety of applications in environmental analysis. Reporter genes are coupled to a sensing element, which recognizes an analyte and thus confers selectivity to the system. The reporter protein produces a detectable signal, which determines the system's sensitivity. The machinery that certain microorganisms have developed to survive in environments contaminated with toxic

metals is conferred by the operons. The operon in turn consists of genes that code for proteins that help bacteria in extruding the toxins out of the cell. The expression of the genes of the operon is tightly regulated by the presence or absence of specific metals in the cells. This metal induced expression can be utilized in developing biosensing systems for the specific toxic metals. The biosensing strategy employed is based on the genetic fusion of the reporter gene to the regulatory gene of the operon induced by its respective metal. Thus, when the induction takes place in the presence of the target metal, the reporter gene is co-expressed along with the other genes of the operon. Consequently, the concentration of the inducer can be quantified by measuring the signal generated by the reporter protein. Based on this strategy, sensing systems for environmental pollutants such as copper, cadmium, arsenite, and antimonite have been developed.

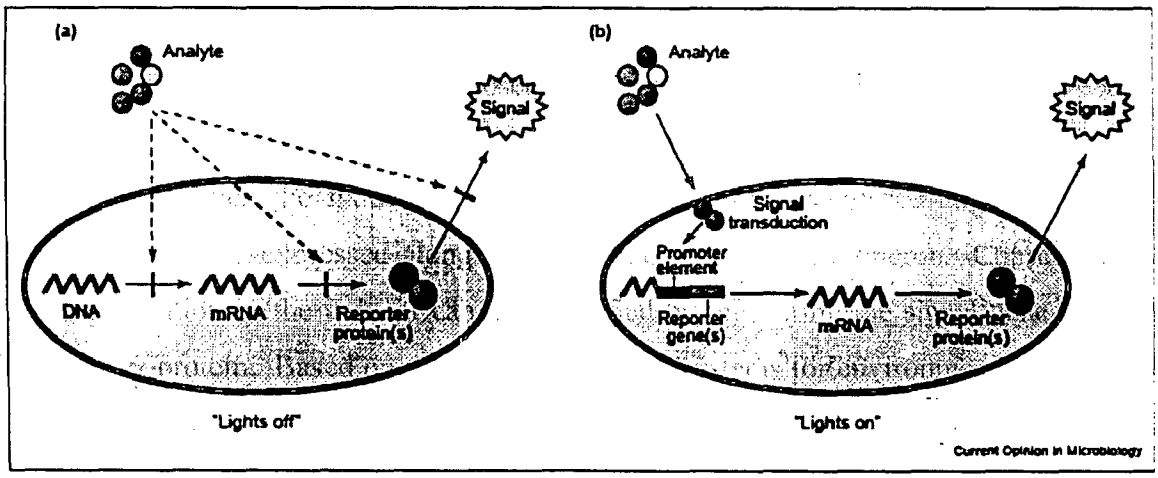


Fig. 2.4- Bacterial toxicity assay principles. In 'lights off' assays (a), sample toxicity is estimated from the degree of inhibition of a 'normally on' activity. Such inhibition can occur along any stage of the reaction's development or in any site affecting cellular wellbeing. In 'lights on' assays (b), a quantifiable molecular reporter is fused to specific gene promoters, known to be activated by the target chemical(s).

Bacteria can be detected using "biosensors." A biosensor is a device that detects, records, and transmits information regarding a physiological change or the presence of various chemical or biological materials in the environment [29]. More technically, a biosensor is

a probe that integrates a biological component, such as a whole bacterium or a biological product (e.g., an enzyme or antibody) with an electronic component to yield a measurable signal. Biosensors, which come in a large variety of sizes and shapes, are used to monitor changes in environmental conditions. They can detect and measure concentrations of specific bacteria or hazardous chemicals; they can measure acidity levels (pH). In short, biosensors can use bacteria and detect them, too.

Genetically engineered bacteria can also be useful because of their ability to "tattle" on the environment. Bacteria have been designed to give off a detectable signal, such as light, in the presence of a specific pollutant they like to eat. They may glow in the presence of toluene, a hazardous compound found in gasoline and other petroleum products [30]. They can indicate whether an underground fuel tank is leaking or whether the site of an oil spill has been cleaned up effectively. These informer bacteria are called bioreporters. Biosensors can detect the presence of biological and chemical warfare agents for military use and for determining the effectiveness of cleaning up waste sites (Fig. 4.1).

Biosensing systems based on whole cells are finding use in a variety of applications in environmental analysis. Reporter genes are often coupled to a regulatory element that recognizes an analyte and confers selectivity to the system, while the reporter protein produces a detectable signal controlling the system's sensitivity. In our work, we employ genetically engineered bacteria-based sensing systems for the detection of environmental pollutants in ground water. Heavy metals such as arsenic and antimony have been shown to have significant effects on human health. Consequently, there is a need for simple analytical techniques to monitor directly the levels of these toxicants. Microorganisms have developed resistance mechanisms in order to survive in environments that are contaminated. The resistance to arsenic and antimony is conferred on bacteria by the ars operon, which codes for proteins that help bacteria in extruding these toxicants out of the cell. We have developed biosensing systems for arsenic/antimony by genetically designing bacteria in a way that couples the natural regulatory mechanisms of Escherichia coli with the expression of reporter genes. In such

systems, the level of expression of the reporter protein can be related to the concentration of the target compound(s) present in the environment. Cell-based biosensing systems have been reported for various environmental pollutants such as antimony, arsenite, cadmium, lead, zinc, and PCBs among others. However, these systems have not yet found field applications despite their simplicity to operate, ease of production, and low cost. Here, we describe the development of a set of bacterial biosensors based on three non-pathogenic laboratory strains. The biosensing systems were genetically improved to reduce background expression in the absence of the analyte. Two strategies to adapt these whole-cell sensing systems for field applications were exploited: an inexpensive sensing strip for pre-screening purposes and a microfluidic platform in the shape of a compact disc for quantitative analysis. The sensing strips are a simple way to detect arsenic contamination without the need of expensive instrumentation and trained personnel. In contrast, the microfluidic platform offers high throughput and multiplexing capabilities. Both systems offer complete portability which allows them to be used as field sensors for measuring bioavailable levels of arsenite/antimonite in the environment. These biosensor cells and protocols offer a realistic alternative method for assessing environmental contamination in potable water.

Biosensors combine the exquisite selectivity of biology with the processing power of modern microelectronics and optoelectronics to offer powerful new analytical tools with major applications in medicine, environmental diagnostics and the food and processing industries.

Biosensors consist of bio-recognition systems, typically enzymes or binding proteins, such as antibodies, immobilized onto the surface of physicochemical transducers. The term immunosensor is often used to describe biosensors which use antibodies as their biorecognition system. In addition to enzymes and antibodies, the biorecognition systems can also include nucleic acids, bacteria and single cell organisms and even whole tissues of higher organisms. Specific interactions between the target analyte and the complementary biorecognition layer produces a physicochemical change which is detected and subsequently measured by the transducer. The transducer can take

many forms depending upon the parameters being measured - electrochemical, optical, mass and thermal changes are the most common.

Cell- Based Sensing Systems or Bioreporters

Whole cells as the sensing element offers some unique advantages compared with isolated proteins, whole cells are often less susceptile to changes in environmental conditions such as pH and temperature and the presence of other solutes. Cell based sensing systems provide a measure of the bioavailability of a given analyte, because the analyte must be transported inside the cells or bound to cellular receptors to produce a response. The bioavailability of certain analytes, such as toxins and pollutants, is an important consideration in decisions regarding remediation of contaminated environment because it is better measure of toxicity than the total analyte concentration. Cell-based sensing systems can either be specific for a certain analyte or can respond broadly to a group of analytes. Fig. 2.4 shows anatomy of bioreporter organism.

One type of sensing system of broad specificity uses a reporter gene as a marker for bacterial cell metabolism. This reporter gene codes for a protein, typically a luciferase, that gives rise to a signal under normal metabolic conditions. When the sensor is exposed to an environment containing toxic ions, the cell metabolism is inhibited, decreasing the reporter enzyme activity. This gives a qualitative estimate of ecotoxicity. The light measured in the cells is related to the toxicity of the environment. In these whole cell sensors, any compound that affects either the cellular metabolism or causes stress in the cell induces a response. This system can be applied to a wide variety of potentially hostile environments to provide initial warning of adverse effects and trigger subsequent analysis and remediation actions [27].

The resistance mechanism of microorganisms to metals and organic compounds is the result of several enzymes and proteins acting in concert to neutralize the toxicity of these compounds. The production of proteins and enzymes that confer this resistance is regulated by a control system made up of regulatory proteins and specific promoter regions of chromosomal or plasmid DNA. The proteins and /or enzymes are expressed

only when the specific compound is present. By inserting a gene for a reporter protein into the plasmid, a signal is produced in response to a specific compound.

Certain strains of bacteria can survive in environments contaminated with arsenite, arsenate, and antimonite because they possess the necessary genetic configuration to make them resistant to these species. Resistance is conferred by the ars operon, which consists of five genes that code for three structural proteins, ArsA, ArsB, ArsC, and two regulatory proteins, ArsR and ArsD. The proteins ArsA, ArsB, and ArsC form a pump system that extrudes antimonite, arsenite and arsenate from the cell once the anions reach the cytoplasm of the bacterium. Arsenite and antimonite trigger the transcription and translation of the genes that constitute the pump proteins. Although all organisms have their own defense mechanism against arsenic but here we are interested in exploiting arsenic resistance mechanism in *Escherichia coli*.

2.2.1 Reporter gene technology

Reporter genes code for proteins that produce a signal that allows a protein to be determined in a complex mixture of other proteins and enzymes. Reporter genes are now being used as part of a signal transduction event in many biosensing systems and have been used to develop assays for such diverse compounds as metal ions, toxic organic species, viruses and antibodies. A molecular recognition event is usually coupled to a reporter event to provide the required sensitivity and selectivity to detect the analyte. The molecular recognition event recognizes the selectivity of the system and reporter event generates a signal which controls the sensitivity of the bioreporter. Reporter proteins can be monitored by a variety of detection systems such as electrochemical, fluorescence, bioluminescence and chemiluminescence .Table 2c illustrates several reporter proteins, and their reactions to produce biological signal. Many organisms having reporter proteins with different emission wavelength have been found e.g., single-celled algae, sea walnuts, jellyfish, fireflies, worms and even some mushrooms are bioluminescent because certain photoproteins or enzymes are present [25].

Several types of reporter genes are available for use in the construction of bioreporter organisms, and the signals they generate can usually be categorized as either

colorimetric, fluorescent, luminescent, chemiluminescent or electrochemical. Although each functions differently, their end product always remains the same – a measurable signal that is proportional to the concentration of the arsenite to which they have been exposed. In some instances, the signal only occurs when a secondary substrate is added to the bioassay (*lux*AB, *Luc*, and aequorin). For other bioreporters, the signal must be activated by an external light source (GFP and UMT), and for a selected few bioreporters, the signal is completely self-induced, with no exogenous substrate or external activation being required (*lux*CDABE).

Table 2c: Reporter Proteins.

Reporter Protein	Reaction	Detection
Chloramphenicaol acetyltransferase from *E.coli*	Acetylation of chloramphenicol or its derivatives	Radioisotope, Fluorescence
Alkaline phosphatase	Phosphate hydrolysis	Electrochemical, Chemiluminescence, Fluorescence
β-Galactosidase	Hydrolysis of β-galactosides	Electrochemical, Chemiluminescence, Fluorescence
β-Glucuronidase	Hydrolysis of β-glucuronides	Electrochemical, Chemiluminescence, Fluorescence
Bacterial luciferase	$FMNH2 + decanal + O_2 \rightarrow FMN +$ decanoic acid + H_2O + hν (490nm)	Bioluminescence
Firefly luciferase	ATP + firefly luciferin + $O_2 \rightarrow$ AMP + oxyluciferin + inorganic phosphate + hν(560nm)	Bioluminescence
Aequorin	Apoaequorin + coelenterazine + O_2 + Ca^{2+} → Apoaequorin + coelenteramide + CO_2 + hν(469nm)	Bioluminescence
Obelin	Apoobelin + coelenterazine + O_2 + Ca^{2+} → apoobelin + coelenteramide + CO_2 + hν(470nm)	Bioluminescence
Green fluorescent protein	Posttranslational formation of an internal chromophore	Fluorescence
Urogen III methyltransferase	Transmethylation of urogen III to precorrin-2 oxidized tosirohydrochlorin and is transmethylated to trimethylpyrrocorphin	Fluorescence

β – Galactosidase (*Lac Z*):

Beta galactosidase is an enzyme that indicates the breakdown of the sugar lactose. Lactose is a type of sugar found in milk. It is composed of two rings. The rings contain 5 carbon atoms, one at each corner and an oxygen atom. Attached to the carbon atoms area a hydrogen atom and an OH group or another carbon.

ß-galactosidase

Lactose ----------> Galactose + Glucose

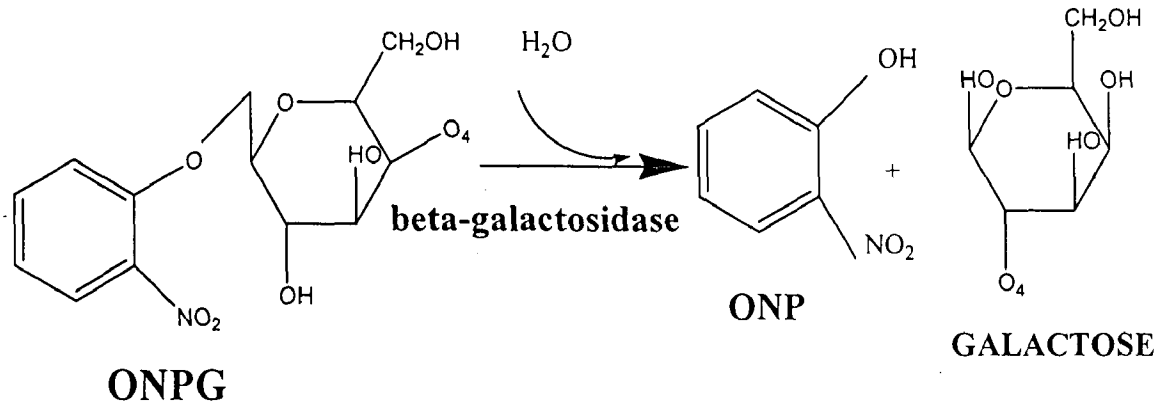
The rings are bound together by an oxygen bridge. This bridge can be broken when the enzyme beta galactosidase binds to lactose and a water molecule reacts with the oxygen atom in the bridge (Hydrolysis reaction). Association of lactose with beta galactosidase facilitates the reaction between water and lactose. This breaks the oxygen bridge and results in the production of two simple sugars (glucose and galactose). Like lactose ONPG (o-nitrophenyl-β-D-galactopyranose) is a molecule composed of two rings held together by an oxygen bridge. Beta galactosidase also forms a complex with ONPG molecules and is able to hydrolyze them.

The amount of o-nitrophenol formed can be measured by determining the absorbance at 420 nm. If excess ONPG is added, the amount of o-nitrophenol produced is proportional to the amount of ß-galactosidase and the time of the reaction. The reaction is stopped by adding Na_2CO_3 which shifts the reaction mixture to pH 11. At this pH most of the o-nitrophenol is converted to the yellow colored anionic form and beta galactosidase is inactivated. The reaction can be run using whole cells that have been permeabilized to allow ONPG to enter the cytoplasm. However, since whole cells are present, the absorbance at 420 nm is the sum of the absorbance due to o-nitrophenol and light scattering due to the cells. The contribution of light scattering can be determined by measuring the absorbance at 600 nm where o-nitrophenol doesn't absorb. The light scattering at 420 nm is 1.75x the light scattering at 600 nm, so the absorbance of o-

nitrophenol is determined by subtracting 1.75 x OD600 nm. The corrected absorbance is then used to calculate the activity of ß-galactosidase.

Enzyme-substrate kinetics

When AW10 bacteria containing pMV-arsR-ABS plasmid are exposed to arsenite, ArsR is released from the plasmid and β-galactosidase is expressed. Thus β-galactosidase gene acts as reporter gene in our system. ONPG (o-nitrophenyl-β-D-galactopyranose) is a molecule composed of two rings held together by an oxygen bridge. B-galactosidase also forms a complex with ONPG molecules and is able to hydrolyze them. Hydrolysis of ONPG is shown below..



Hydrolysis of ONPG by the action of β-galactosidase enzyme.

The amount of o-nitrophenol formed can be measured by determining the absorbance at 420nm. If excess ONPG is added, the amount of o-nitrophenol (ONP) produced is proportional to the amount of β-galactosidase and the time of the reaction. We measured the level of β-galactosidase (β-gal) indirectly- as a function of its enzymatic activity on the lactose analog o-nitrophenyl-β-galactopyranoside (ONPG) [26].

$$ONPG + \beta - gal \xrightarrow{k_1} ONP + galactose + \beta - gal \qquad (1)$$

β-galactosidase is an enzyme and thus appears on both sides of the equation. Assigning rate constants to each step, we get

$$\frac{d}{dt}[ONPG] = k_{-}[ONP][galactose][\beta - gal] - k_{+}[ONPG][\beta - gal] \quad (2)$$

$$\frac{d}{dt}[ONP] = k_{+}[ONPG][\beta - gal] - k_{-}[ONP][galactose][\beta - gal] \quad (3)$$

Now by taking the assumption, that backward reaction has a much lower rate than the cleavage rate ($k_{-} << k_{+}$) and that we have saturating concentrations of ONPG.

$$\frac{d}{dt}[ONP] = k_{+}[ONPG][\beta - gal] \quad (4)$$

$$\frac{d}{dt}[ONPG] = -k_{+}[ONPG][\beta - gal] \quad (5)$$

Under these assumptions, observing equation (4) we expect a linear increase in the rate of ONP production as a function of [β-gal]. The ONP production is measured by atomic absorption spectrophotometer.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents

Solutions were prepared using deionized (Milli-Q Water Purification system, Millipore) distilled water. All chemicals were reagent grade or better and were used as received.

3.2 Strain and plasmid

E.coliDH5α containing the plasmid pMV132-arsR-ABS as shown in Figure 4.3, a gift from Dr. Jan Roelof Van Der Meer of Swiss Federal Institute for Environmental Science and Technology. Plasmid pMV132-arsR-ABS had Ampicillin resistance genes and was isolated from this bacterial biosensing system. AW10 strain of *E.coli* as shown in Figure 4.4 was gifted by Professor Barry P. Rosen of Wayne state University School of Medicine which had chloramphenicol resistance genes in the chromosomes of the cell. So, the bacterial biosensing system mAW10 was resistant to both Ampicillin and chloramphenicol.

3.3 Isolation and transformation of the plasmid

The plasmid pMV132-arsR-ABS was isolated from *E.coliDH5α* and transformed into AW10 strain of *E.coli* cells using conventional protocols [36].

3.4 Apparatus

Bacterial cells were grown in an Orbital Shaker/ incubator (Metrex Scientific Instruments Pvt. Ltd., India). Chemiluminescence measurements were taken in Single beam Microprocessor Scanning visible spectrophotometer (Model TVS-25A, Toshniwal Process Instruments Pvt. Ltd., India) using glass cuvettes (Toshniwal Process Instruments Pvt. Ltd., India). Chemiluminescence intensities reported are the average of a minimum of three replicates and have been corrected for the contribution of the blank. All molecular biology procedures were performed using standard protocols [36].

3.5 Dose-response curves for arsenite using mAW10.

mAW10 were plated on LB agar with 100 μg/mL Ampicillin, 25 μg/mL of chloramphenicol and X-GAL. The colony pale blue in color is inoculated in 100mL of LB medium containing 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol at 37 °C until it reached an optical density value of 0.6 at 600 nm (OD_{600} of 0.1 = 1X 10^8 cells/mL). Assay mixtures contain: 2 mL cell suspension and 2 mL sample (or arsenite stock solution). Calibration series vary over the range 0.05-9 μM arsenite (end concentration). Arsenite solutions were prepared fresh daily by serial dilution using deionized water from a freshly prepared 1 X 10^{-3} M stock solution. Mixtures were incubated at 37 °C in a rotary shaker (190 rpm) for 30 min to induce protein expression. After 1h, 20 μL of aliquot of assay mixture is removed for the LacZ assay (remainder is kept on ice for OD_{600}) and added to 80 μL of permeabilization solution (100 mM dibasic sodium phosphate (Na_2HPO_4), 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL CTAB

(hexadecyltrimethylammonium bromide), 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL beta-mercaptoethanol) into 1.5 mL microfuge tubes. After the last sample is over 600 µL of substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/mL o-nitrophenyl-β-D-galactoside (ONPG), 2.7 µL/mL β-mercaptoethanol) is added to each tube at 30 °C room temperature [37, 38]. It was observed that after 25 minutes sufficient color developed, so 700 µL of stop solution (1M Na_2CO_3) is added and mixed well. After stopping the last sample microfuges were spinned in the centrifuge for 5 min at 13,000 rpm. Solutions were transferred carefully in to the cuvette avoiding particulate mater in the cuvette so that scattering does not influence the OD_{420} reading. Activity of beta-galactosidase enzyme was calculated by following equation:

Calculation of beta-galactosidase activity in Miller Units:

$$1\,Miller\,Unit = \frac{1000\,X\,OD_{420}}{OD_{600}\,X\,0.02\,X\,25} \quad (1)$$

3.6 Selectivity studies

Strain mAW10 was used to evaluate the selectivity of the bacterial system toward arsenate, Cd^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}. The standard solutions of the ions were prepared fresh, daily from a freshly prepared 1 X 10^{-3} M stock solution. A volume of 100 µL of the standard solution was incubated for 30 min with 100 µL of the bacterial stock, in order to induce protein expression. The cell membranes were disrupted by permeabilization solution which contains detergent, while the β-galactosidase enzyme remains intact and the chemiluminescence was measured by using the protocol described above.

3.7 Comparison of plasmid stability in two different strains with pMV132-arsR-ABS.

mAW10 were plated on LB agar with 100 μg/mL Ampicillin and X-gal (Fig. 4.17). The colony pale blue in color were inoculated in five 250mL flasks containing 100mL of LB medium along with 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol and were incubated at 37 °C at 120 rpm. Also, at the time of inoculation As(III) was added in each flask containing media to make its final concentration in solution as 0,20,40,60,80 ppm. Similarly, *E.coliDH5α* harboring the plasmid pMV132-arsR-ABS was plated on LB agar with 100μg/mL Ampicillin and X-GAL. The colony pale blue were inoculated in five 250mL flasks containing 100mL of LB medium along with 100 μg/mL ampicillin. At the time of inoculation As(III) was added in each flask containing media to make its final concentration in solution as 0, 20, 40, 60, 80 ppm. After an interval of 24hrs samples was taken out for determining OD at 600nm and also for cell counting on selective and non-selective plates. The number of plasmid –bearing cells was determined by dilution and plating technique. Culture samples were appropriately diluted in saline buffer (9% w/v NaCl) and plated onto six LB agar plates (two plates with antibiotics and two without antibiotics and two X-gal plates with antibiotics) were taken [13]. The dilution factor was decided on the basis of the total number of colonies, which could be conveniently counted (150 to 300). Diluted samples were plated asceptically on the agar plates. After spreading the sample, the agar plate was incubated at 37°C for 16 h. After incubation, the numbers of colonies in each plate were counted and average of two similar plates was calculated.

The plasmid containing fraction of the population was determined by replica plating from selective to non-selective medium for each sample taken at 24 h interval was calculated and colorimetric plate assay was used to detect mutation in the plasmid product. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is a substrate of the *lacZ* gene product (β-galactosidase), and turns blue when cleaved by this enzyme.

3.8 Indirect method for arsR expression for various arsenite concentrations

The enzyme β-galactosidase is a stable and easily assayed in liquid culture by ONPG tests. As *lacZ* gene is in downstream to *arsR* gene in the plasmid, ONPG test was conducted to indirectly determine the expression of *arsR* gene in plasmid. ONPG test for β-galactosidase enzyme assaying was conducted by following Modified Miller method [37,38]. It was observed that after 25 minutes sufficient color developed, so stop solution (1M Na_2CO_3) is added and mixed well for mAW10 strain during ONPG test. Miller units were calculated as

$$1\,Miller\,Unit = \frac{1000\,X\,OD_{420}}{OD_{600}\,X\,Volume(mL)\,X\,25(t,\min)}$$

3.9 Plasmid stability of mAW10 strain at various arsenite concentrations

Three 250 mL flasks containing 100mL L.B. broth were inoculated by pale blue color mAW10 cell colony and kept for shaking in incubator at 37°C at 120 rpm. After 12 h, sample was taken out for OD_{600} and after finding them in late exponential growth phase, arsenite was added to each of the flasks (20ppm, 40ppm, 60ppm respectively). Now, plasmid stability was determined by using the protocol described above.

4.0 Plasmid stability in Immobilized reactor

An 80cm high cylindrical glass column with internal diameter 2.5cm was filled with autoclaved GAC (2-4mm) in laminar hood, and all its connections were made airtight to prevent contamination. All precautionary measures were taken to avoid contamination of any kind. A sintered glass filter disk of 70μm pore size was fitted at the bottom of the cylindrical glass column. mAW10 strain was grown in L.B. broth under selection pressure of antibiotic, when the OD at 600nm was about 1.473 (late exponential phase) the cells were immobilized in the reactor by recycling the culture twice through the reactor. Than a continuous supply of L.B. broth along with arsenite concentration of 60ppm was supplied at a constant dilution rate of 0.02 L/min. The reactor was maintained at a constant temperature by using an automatic heater set at 37°C in a closed chamber Fig. 4.14. Samples were taken out through sterile disposable injections at an interval of 24 hours and plasmid stability was calculated by the protocol described above.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Design of pMV132-arsR-ABS

The design of the biosensors is generally based on cells or molecules that can produce a detectable response upon sensing a change in the metal content of the environment [42]. To avoid the background expression of the reporter gene, *LacZ* gene here, two arsR binding sites have been introduced at either side of the *arsR* , which restricts to the production of *arsR* mRNA as background. In this design at genetic level ArsR binds to both DNA binding sites and prevents RNA polymerase from reading the reporter gene. But in the presence of arsenite, ArsR looses its affinity and expression from the reporter gene gets initiated [35]. The chemiluminescence substrates cannot permeate the E.coli cell wall, and therefore, in order to monitor the enzymatic activity, the cell membrane has to be made permeable. In our work, we have disrupted the cell membrane by permeabilization solution which contains detergent, while the β-galactosidase enzyme remains intact and the chemiluminescence was measured by using the protocol described above.

4.2 Construction of Dose response curve

A dose-response curve was constructed after inducing the expression of β-galactosidase in mAW10 strain of bacteria with various concentrations of arsenite during a 30 min incubation time (Figure 4.15). In this system, the bacteria senses the intracellular concentration of arsenite, which in turn is related to the extracellular

concentration of the anion. The time of induction of 30 min was chosen because it yields a sufficiently high chemiluminescence signal already shown by Sridhar Ramanathan et al., [43]. Figure 4.5 shows that the intensity of the chemiluminescence signal obtained is dependent on the concentration of arsenite present in the sample. Based on the dose response curve obtained, arsenite can be detected at extracellular concentrations as low as 0.05 μM and extending to a long concentration range of 9 μM, covering the maximum contaminant level prescribed by EPA. As this mAW10 strain of *E.coli* lacks chromosomal *ars* operon, a linear-response in miller units was observed in the dose-response curve with increasing concentration of arsenite compared to that biosensor by Stocker et al., [35].

4.3 Selectivity of mAW10

As the concentration of arsenite increases, more of the reporter enzyme is expressed resulting in an increase in chemiluminescence signal. The ArsR protein has a binding site that is very specific towards arsenite along with antimonite, which has similar chemical properties as that of arsenite. We did not find any significant response with increasing concentration of soft metal ions such as Cd^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} using mAW10 strain as shown in Figure 4.7 This indicates high selectivity of the ArsR protein, which restricted any of the soft metal ions to induce β-galactosidase enzyme in the strain. This biosensor is also sensitive to the antimonite and its response with antimonite concentration is shown in Figure 4.6.

4.4 Non-Existence of Structural Instability

It was observed that all the colonies on X-gal plates containing antibiotic were blue in color, thus verifying that mutation in plasmid genes had not occurred and also structural instability does not exist to such an extent to get notified. Structural instability originates from changes in the plasmid itself such as point mutation, deletion, insertion and rearrangement in the plasmid DNA [19]. The resulting cells become non-productive.

4.5 Reduced Basal expression – Reduced metabolic burden

The plasmid pMV132-arsR-ABS was specially designed for reduced basal expression (Fig. 4.3)[35]. Basal transcription in the absence of inducer is minimized through the presence of a suitable repressor. ArsR controls the basal level of protein expression. In the absence of arsenite, the ArsR repressor binds to operator/promoter site and prevents expression of proteins. When arsenite enters the cell, it interacts with the ArsR repressor leading to a conformational change and dissociation of the ArsR protein from its operator. Since the promoter/operator site are upstream of *arsR* itself, a basal level expression of *arsR* is required for the system to function or otherwise no arsR would be produced. Also the transcription of downstream genes is to be stopped as basal expression, so a second binding site had been introduced in pMV132-arsR-ABS to reduce the background expression of further downstream genes [35]. Minimization of basal transcription is especially important when the expression target introduce a cellular stress situation and thereby selects for plasmid loss.

4.6 High plasmid stability – Late exponential growth phase

At the onset of exponential phase, the cell synthesizing machinery gets engaged in the primary metabolism and growth associated functions of the host cells, and therefore, the plasmid content gets reduced. While in the stationary phase, cells just maintain themselves, thus the synthesizing capacity of cells gets diminished [40,41]. But Cells grew sufficiently at the late exponential growth phase, so the available translational machinery was mostly utilized for plasmid encoded *arsR* gene expression as well as for expression of *lacZ* gene. So, high plasmid stability was found in late exponential growth phase for every concentration of arsenic (Figure 4.8). It was seen that there was early transcription of plasmid pMV132-arsR-ABS in mAW10 strain to survive in adverse arsenic environment, and therefore, plasmid stability decreased to a great extent in comparison to *E.coliDH5α* harboring the same plasmid but increased at fast pace in late exponential phase. Plasmid stability did not increase as rapidly as mAW10 strain in *E.coliDH5α* at late exponential phase.

4.7 Quantifying ArsR

A rough idea of the quantity of ArsR protein produced by the cell at various concentrations of arsenite can be estimated by indirectly measuring β-galactosidase activity, as *LacZ* gene is present downstream to *arsR* gene in plasmid pMV132-arsR-ABS (Figure 4.9).

4.8 Effect of arsenite on Plasmid Stability

After arsenite was added at various concentrations into mAW10 culture at late exponential growth phase, at first due to a sudden load on cell for plasmid transcription the plasmid stability decreased and then the stability increases. Plasmid survival relies heavily on the regulation of gene expression assuring the balance between the necessity of a certain level of plasmid genetic information being expressed and minimization of the metabolic burden imposed on the host. It has been found that high stability was achieved at high concentrations of arsenite (Figure 4.10).

Introduction of inducer to cells for transcription of plasmids along with inoculation in fresh media reduced stability suddenly to a great extent initially but later stability improved with passage of time. Where as, when induction of plasmid was done in late log phase, plasmids kept high stability due to external environmental pressure of toxic arsenite, forcing the cell to maintain the production of plasmid DNA for its resistance to arsenite.

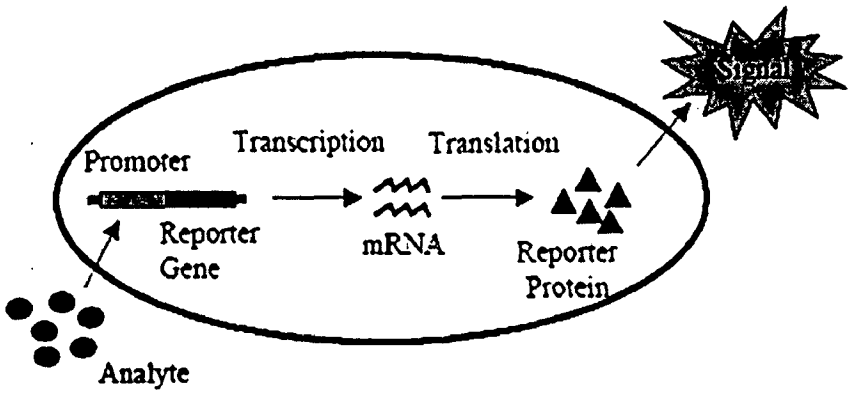


Fig 4.1 Analysis of an analyte by a whole cell Biosensor.

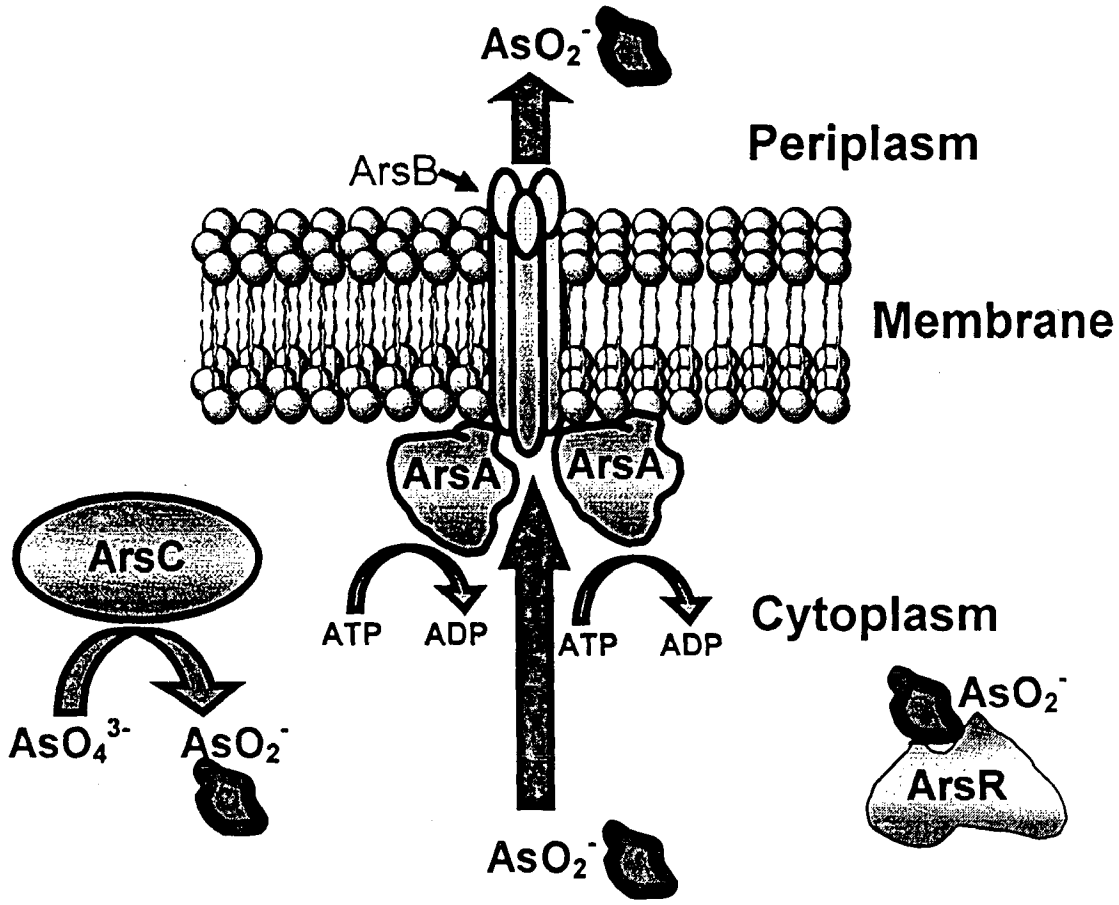


Fig 4.2 Arsenic resistance mechanism.:Arsenate (As(V)) is taken up by phosphate transporters, and As(III) is taken up by aquaglyceroporins (GlpF in *E. coli*, and arsenate is reduced to arsenite by the bacterial ArsC enzyme. Glutathione and glutaredoxin serve as the source of reducing potential. In *E. coli*, arsenite is extruded from the cells by ArsB alone or by the ArsAB ATPase[22].

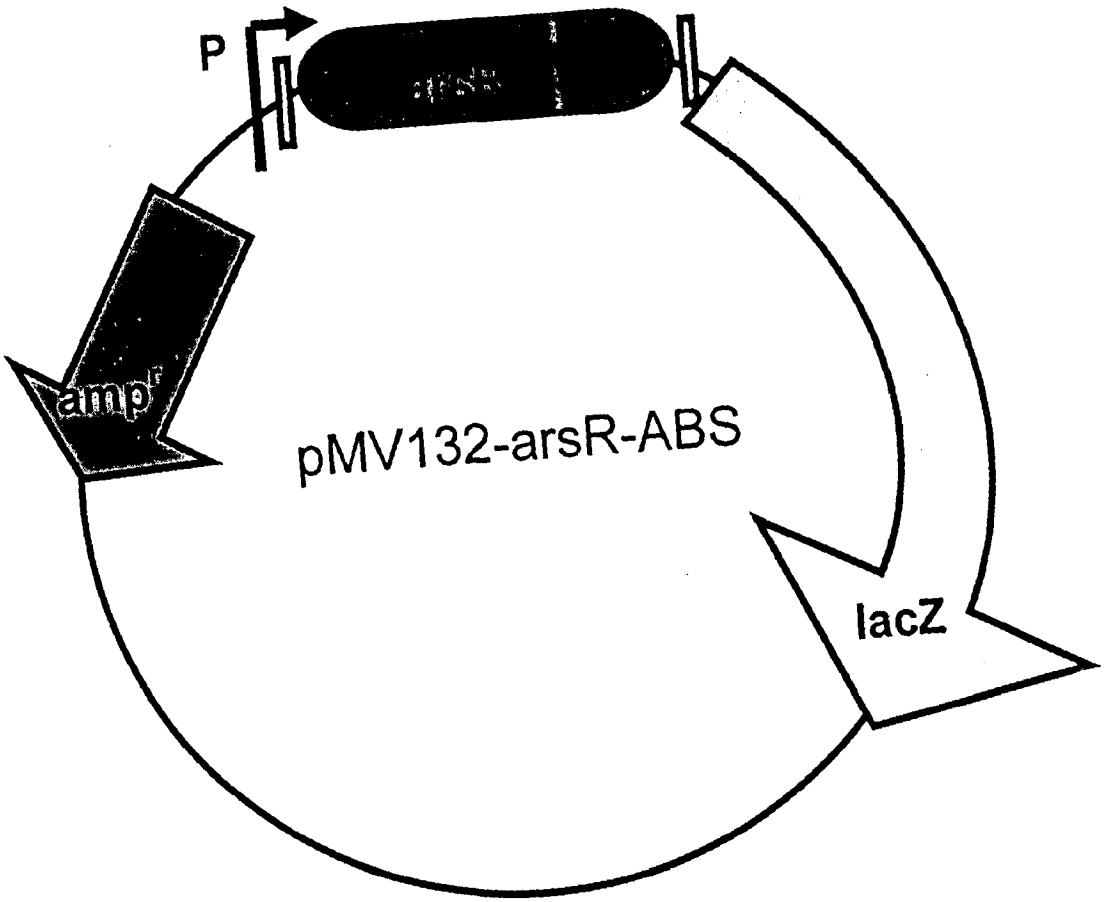


Fig 4.3: Plasmid pMV132-arsR-ABS. Schematic drawing of plasmid pMV-arsR-ABS surrounding with two arsR DNA binding sites[35].

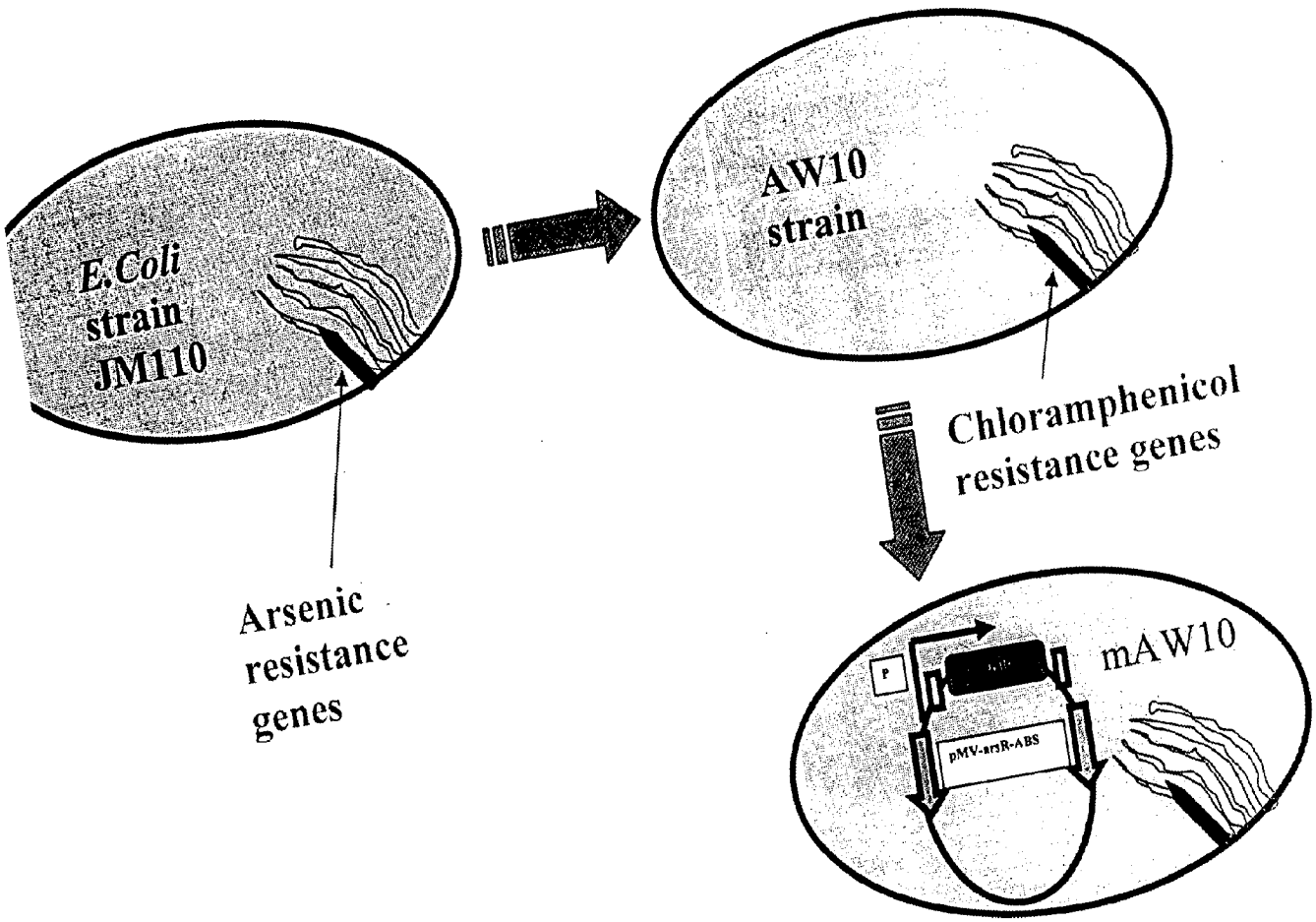


Fig 4.4: Development of mAW10. Development of mAW10 strain of *E.coli* from *E.coli* strain of AW10 which had been already developed by JM110 strain of *E.coli* by deleting the chromosomal *ars* operon and inserting the gene coding for chloramphenicol resistance in its place [43].

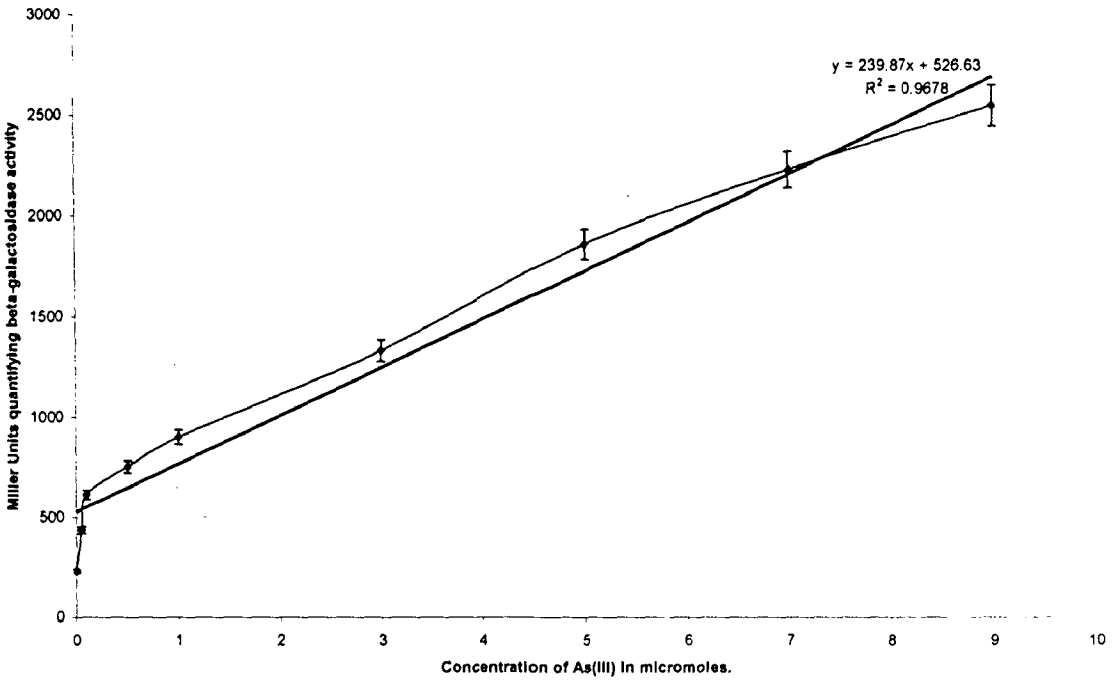


Fig 4.5 Calibration Curve of arsenite. Dose-response curve for arsenite performed after mAW10 were incubated with sodium arsenite for 30 min. The X-axis reflects the arsenite concentration in the sample. Miller units were calculated for various values of optical density obtained. Chemiluminescence intensities obtained was integrated over a period of 3 seconds and has been subtracted from that of the blank. Data are the mean standard deviation of three independent experiments.

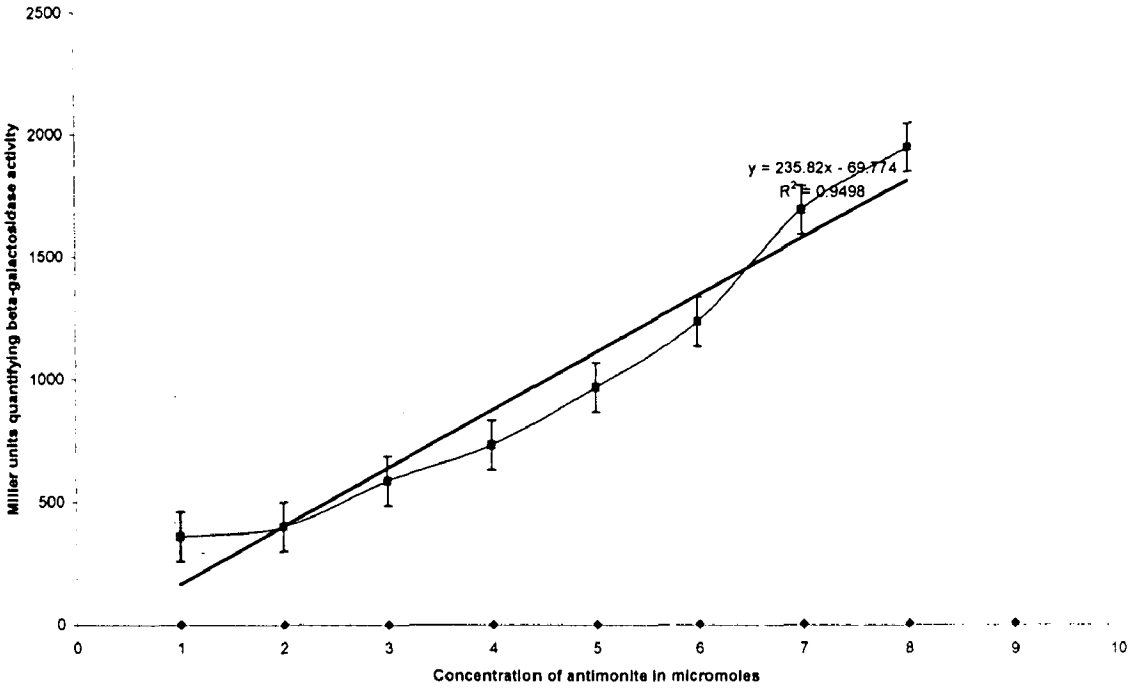


Fig 4.6 Calibration curve of Antimonite. Dose-response curve for antimonite performed after the bacteria mAW10 were incubated with potassium antimonyl tartrate for 30 min. The X-axis reflects the arsenite concentration in the sample. Miller units were calculated for various values of optical density obtained. Chemiluminescence intensities obtained was integrated over a period of 3 seconds and has been subtracted from that of the blank. Data are the mean standard deviation of three independent experiments

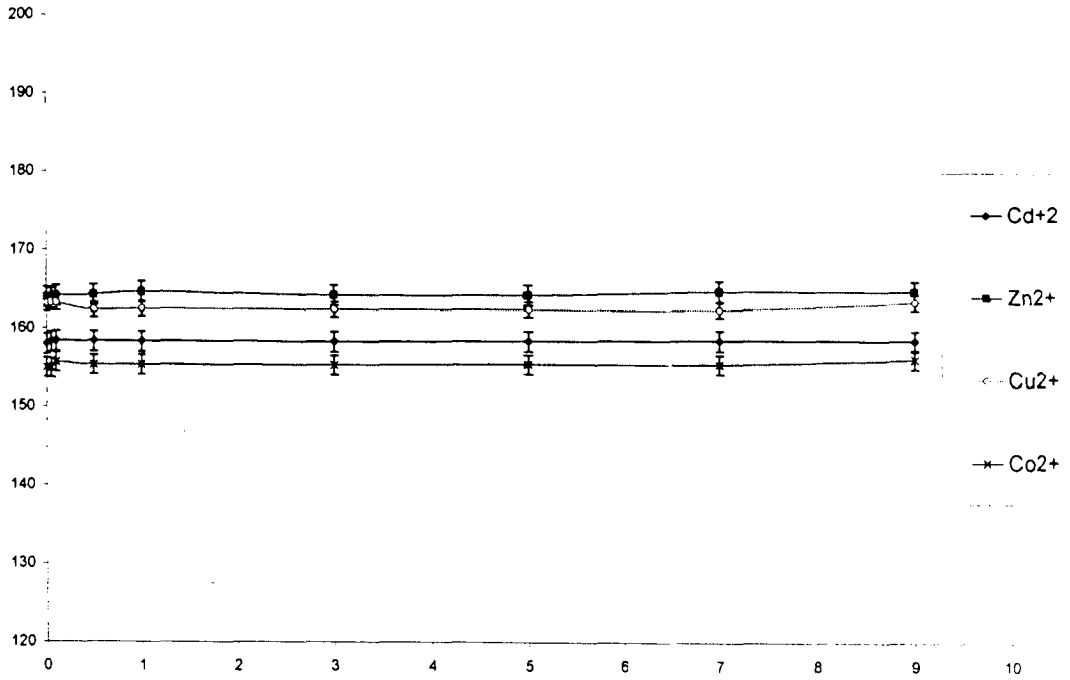


Fig 4.7 Response to other metals. Dose response curves for various metals after the bacteria mAW10 were incubated with various metals for 30 minutes. The chemiluminescence intensities obtained was integrated over a period of 3 seconds and has been subtracted from that of the blank. Data are the mean standard deviation of three independent experiments.

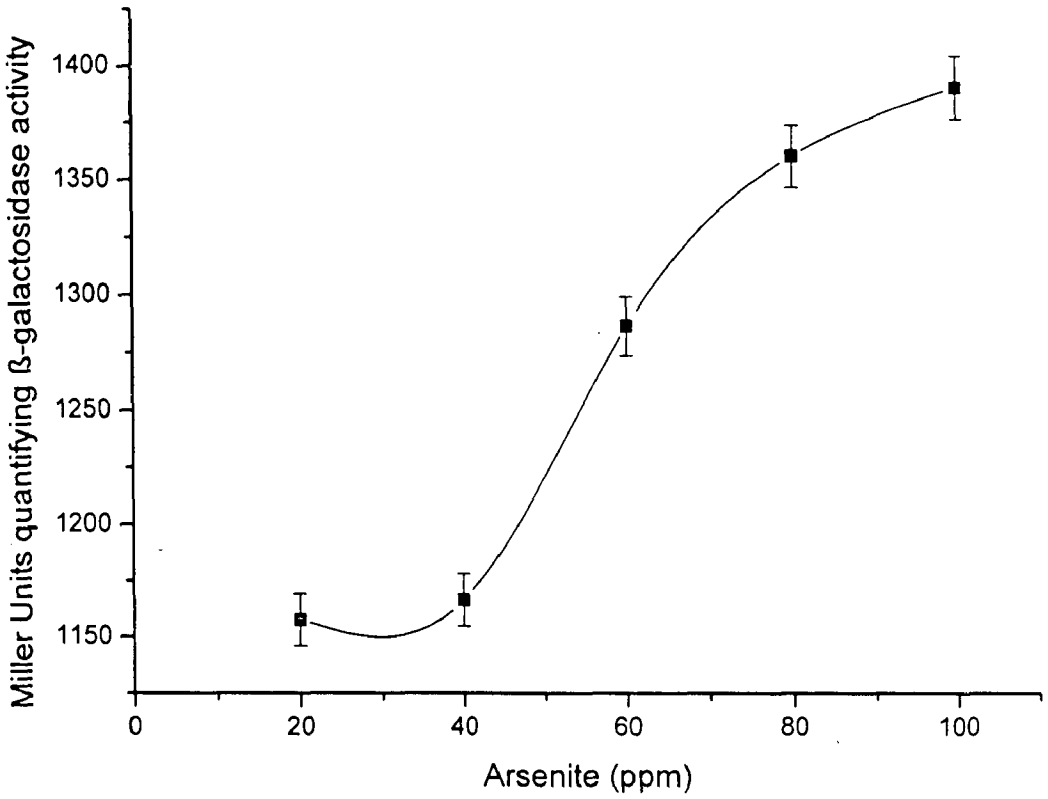


Fig 4.9 Measurement of arsR protein by ONPG test. Dose-response curve for arsenite performed after the bacteria mAW10 were incubated with sodium arsenite for 30 minutes. The x-axis reflects the arsenite concentration in the sample. Miller units were calculated for various values of optical density obtained. Data are the mean standard deviation of three independent experiments

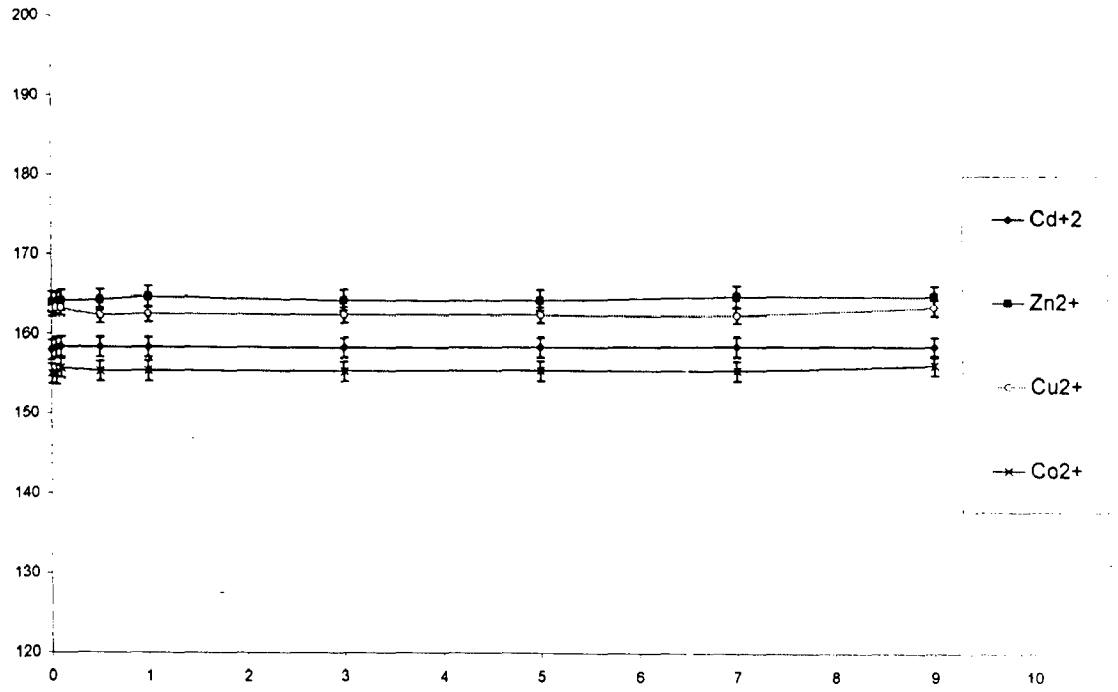


Fig 4.7 Response to other metals. Dose response curves for various metals after the bacteria mAW10 were incubated with various metals for 30 minutes. The chemiluminescence intensities obtained was integrated over a period of 3 seconds and has been subtracted from that of the blank. Data are the mean standard deviation of three independent experiments.

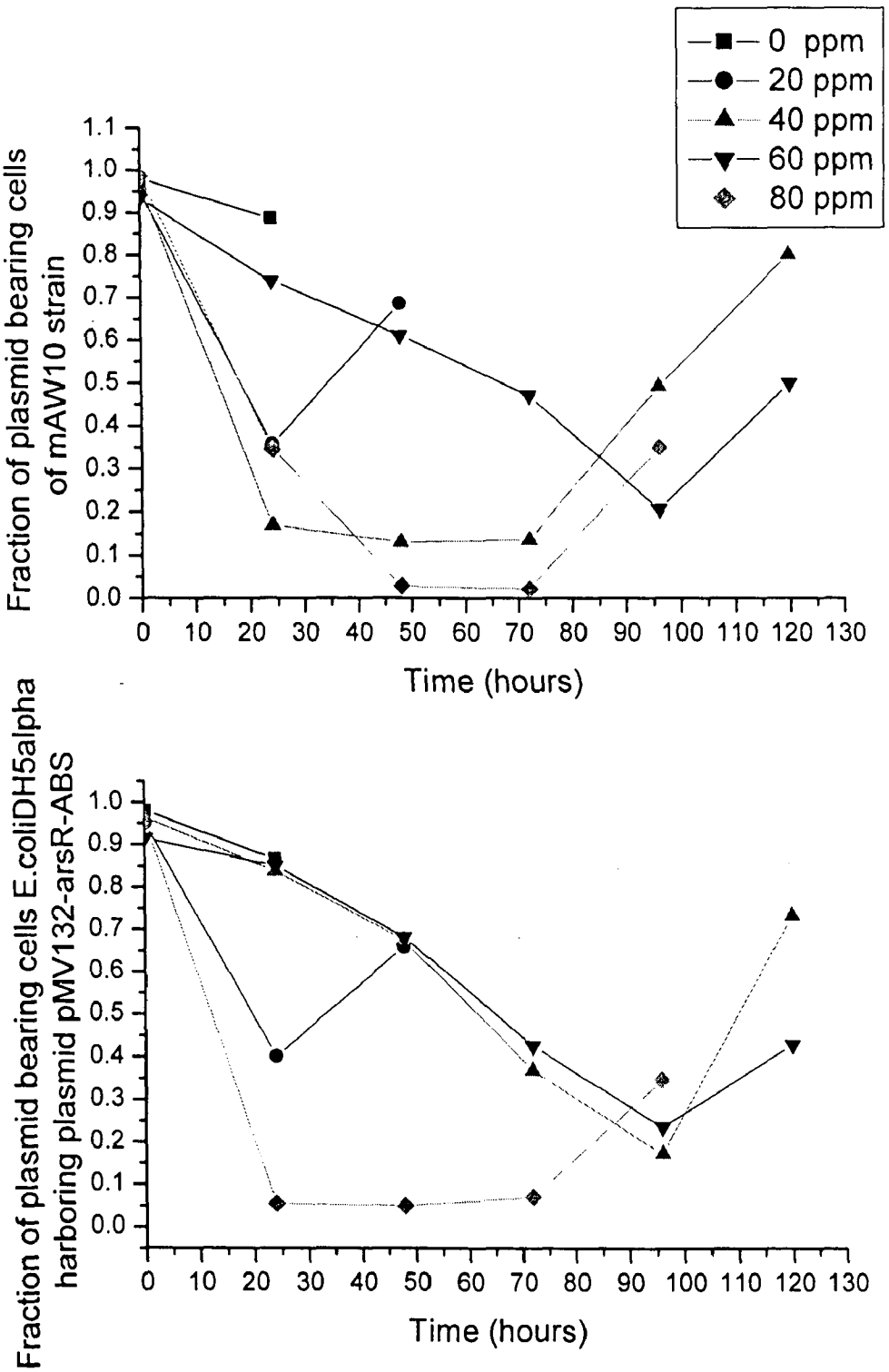
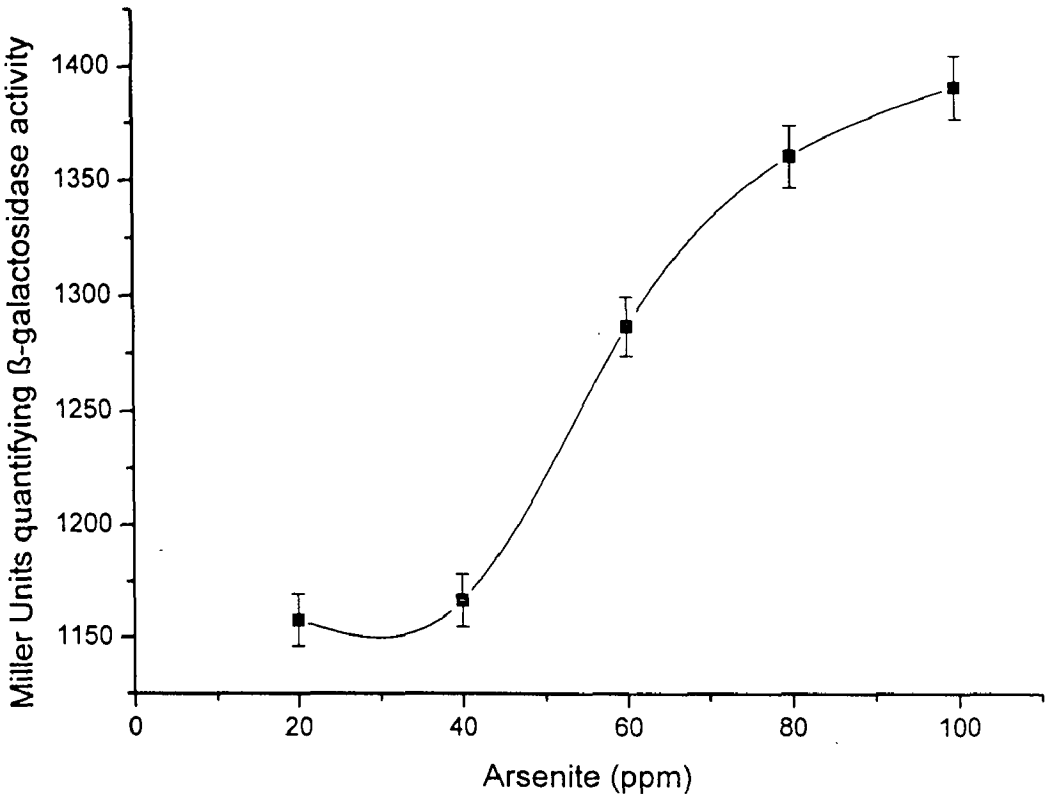


Fig 4.8 Variation of Plasmid stability with growth phase. Comparison of plasmid stability of mAw10 and *E.coliDh5α* strain harboring the plasmid pMV132-arsR-ABS at three different phases of growth with time course when induced by arsenite during inoculation



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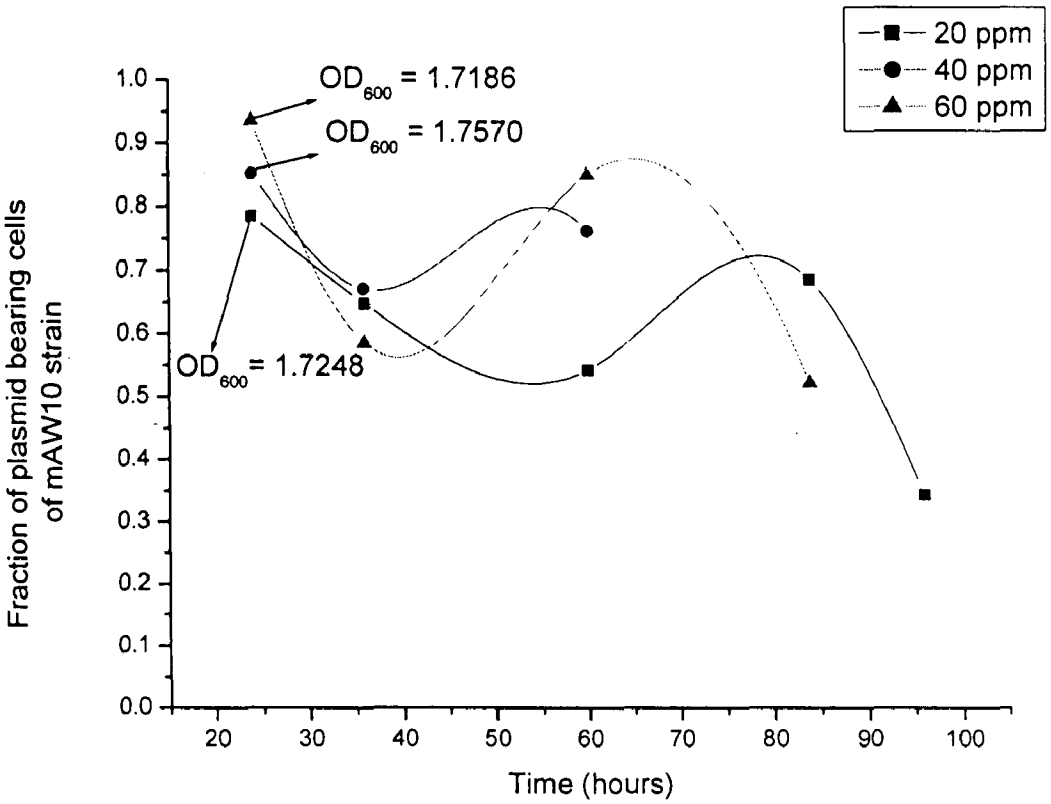

Fig 4.10 Stability at Late-exponential growth phase. Time trajectory of plasmid fraction in mAW10 strain induced at late exponential phase at various arsenite concentrations.

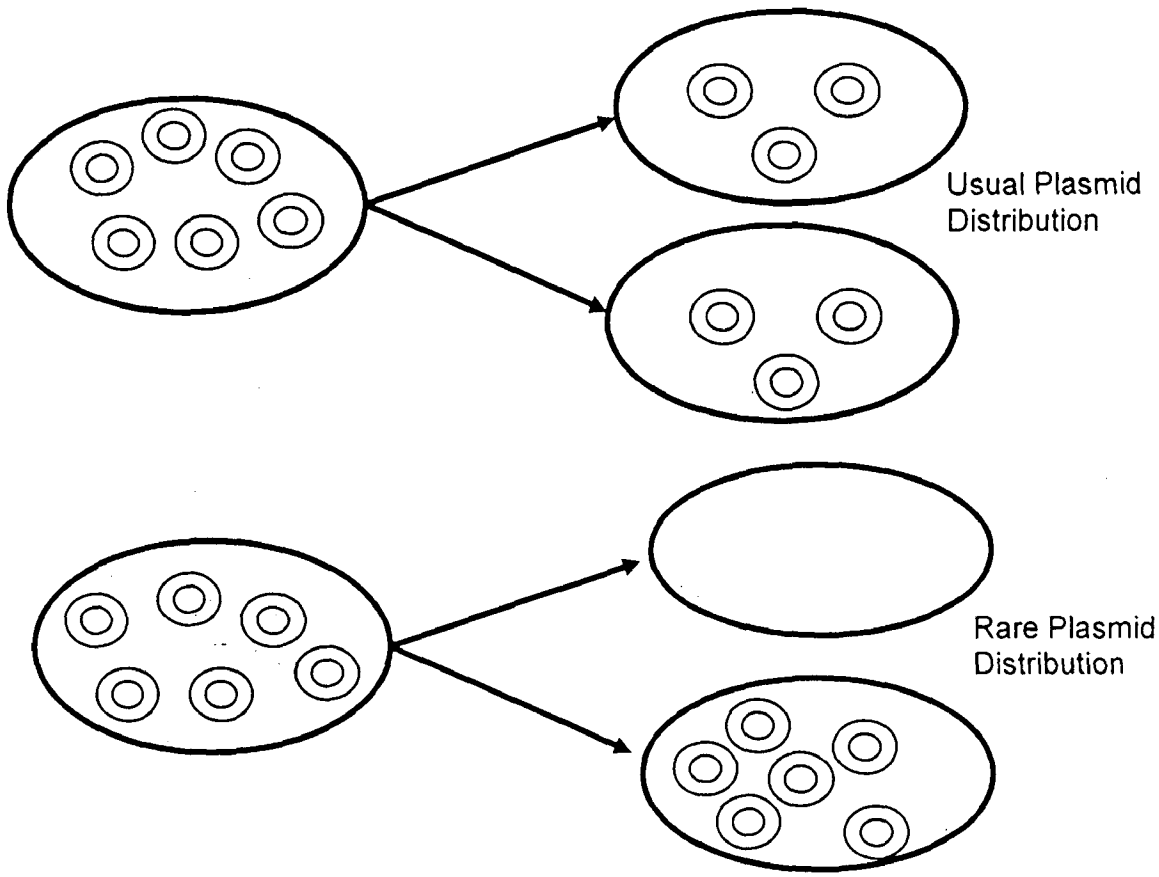

Fig 4.11 Segregational Loss: Segregational Instability results when a dividing cell donates all its plasmids to one progeny and none to the other.

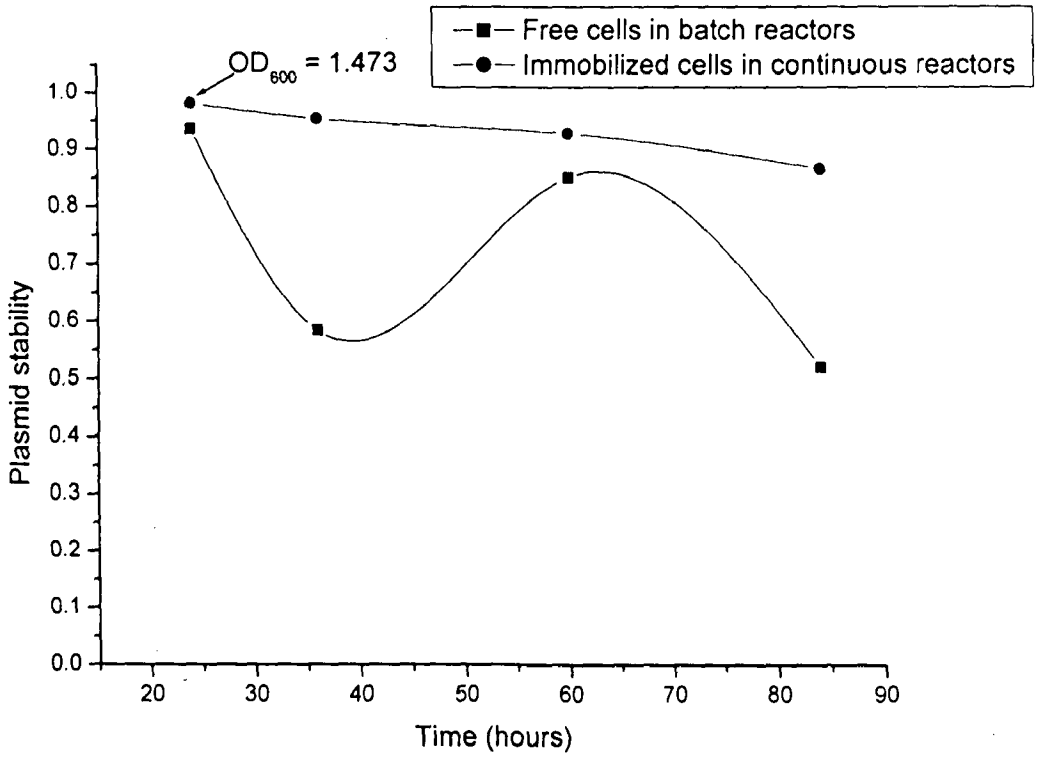

Fig 4.12 Plasmid stability by free and immobilized cells.: Plasmid stability of AW10 strain in Batch reactor and immobilized reactor.

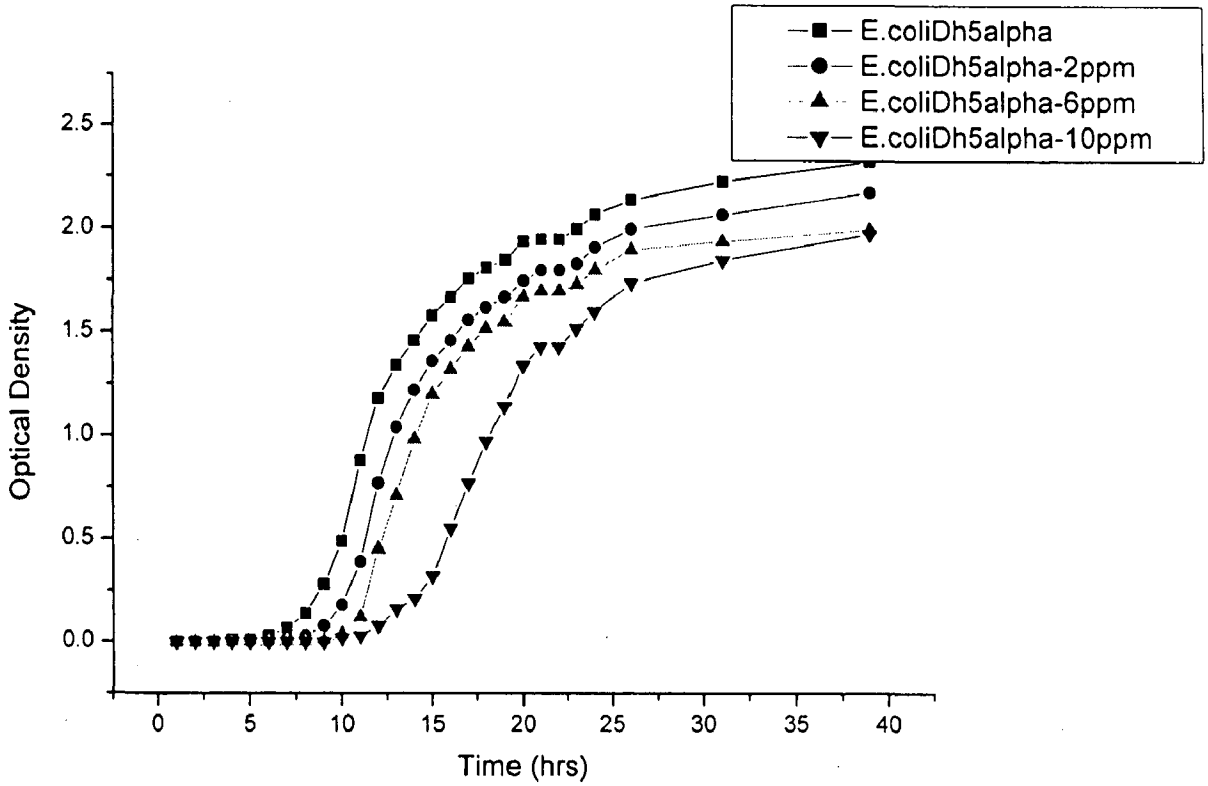

Fig 4.13 Acclimatization of E.coliDH5α to various arsenite concentrations.

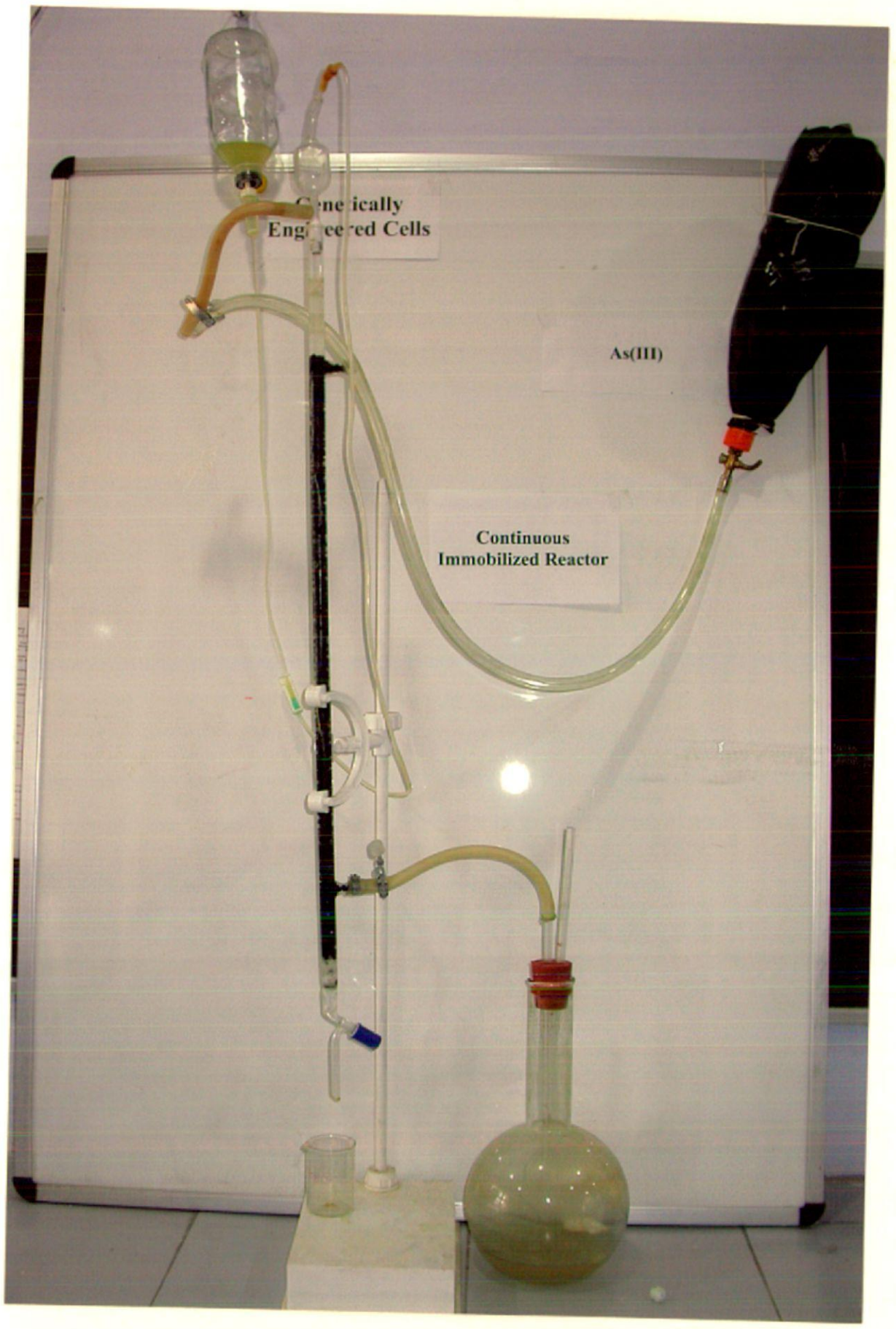

Fig 4.14 Laboratory set up of Continuous Immobilized Reactor.

Fig 4.15 Some results of Enzyme assaying by ONPG test by Modified Miller Method.

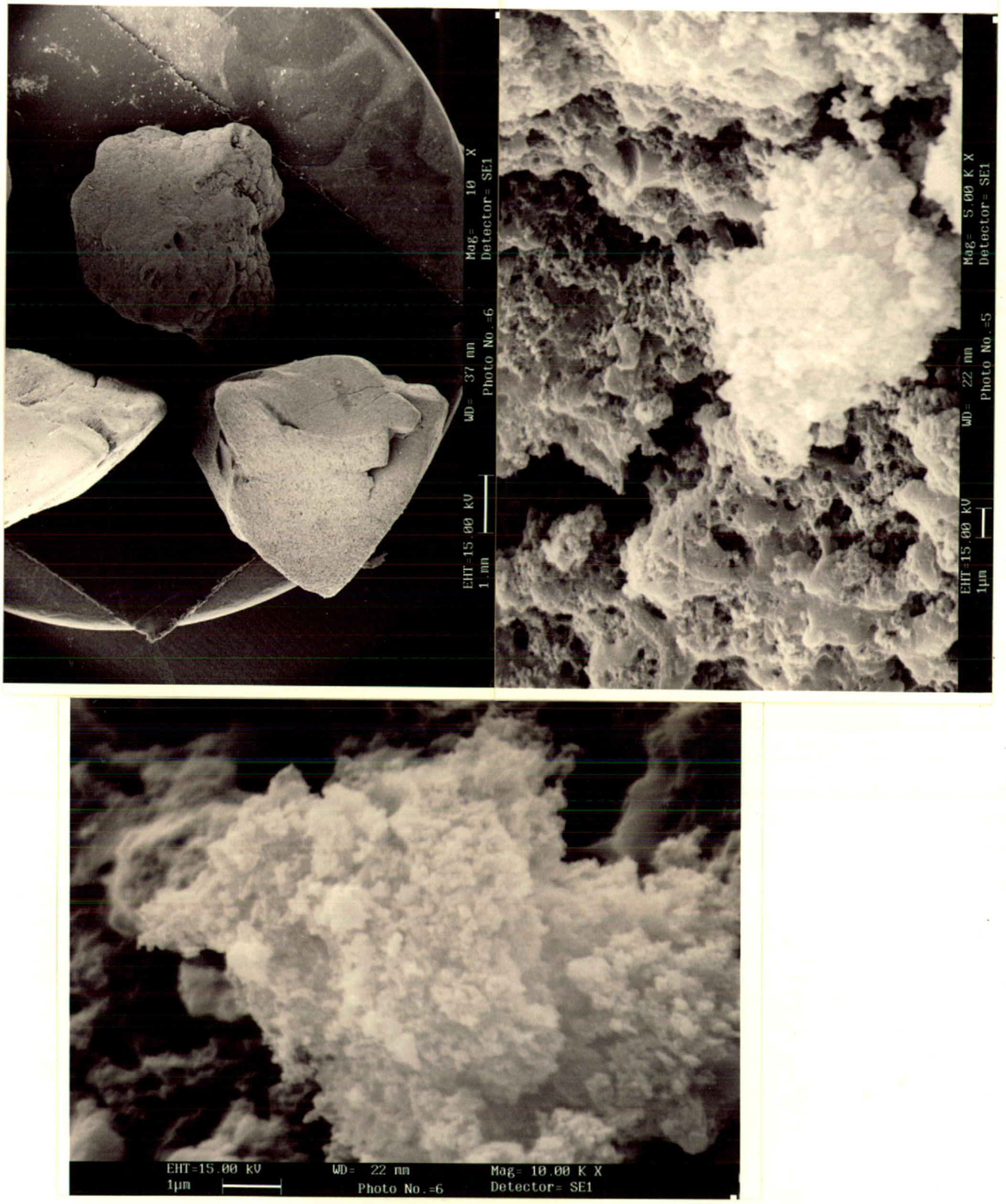

Fig. 4.16 Showing SEM images of Immobilization of cells on GAC.

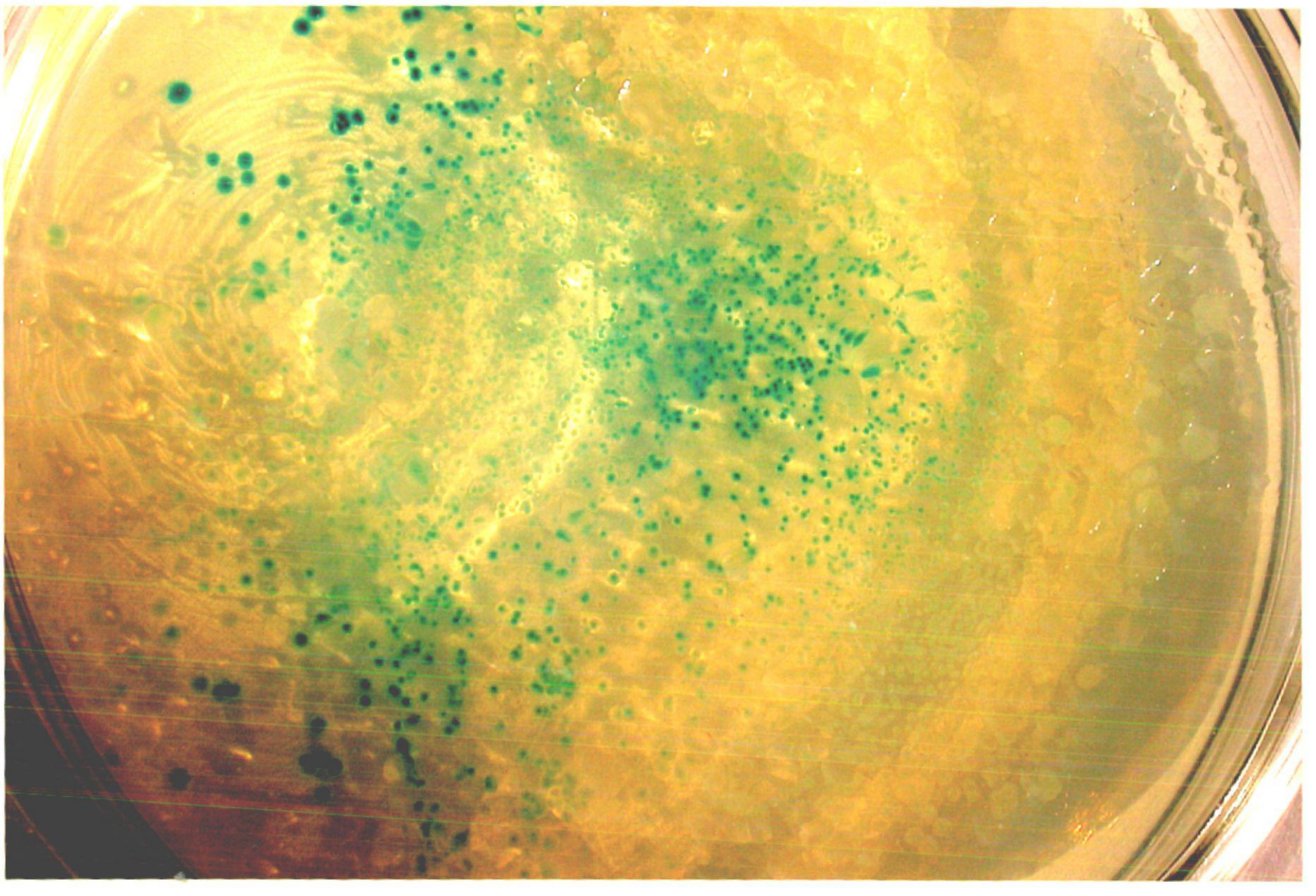

Fig 4.17 Growth of arsenic induced mAW10 on X-gal plates.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSIONS

In summary, we have developed a chemiluminescence sensing system for arsenite using genetically designed bacteria. When the *E.coli* strain of mAW10 is exposed to arsenite anion, β-galactosidase in an amount related to the concentration of arsenite present in the solution is expressed. Using this sensing system, arsenite can be monitored at concentrations as low as 0.05 μM and extending to a higher range covering EPA-proposed maximum contaminant level limit for various countries. Finally, we have arrived to the conclusion that this system is highly selective to arsenite over a variety of metals ions and gives the dose-response curve linear with coefficient of regression R^2 = 0.9678, for a long range of arsenite concentrations 0.05 - 9 μM. Because of the improved detection limits and relatively rapid response time in comparison to already developed arsenite biosensors and due to linear response to bioavailable arsenite, our developed biosensor proves to find applications in environmental monitoring of Arsenite in drinking water. It can be used for easy determination of As (III) concentration in underground water, which is used mainly for the purpose of drinking by the poor villagers without any pretreatment. Easy analysis may help the villagers to test the water by themselves and prevent the use of contaminated water wells.

Our work will promote bioremediation by using recombinant cells, which will attract environmentalist due to cost saved to maintain plasmid in cells by using an alternate selective pressures than antibiotic. Combining this strategy together with appropriate genetic and environmental conditions for the host, may lead to host-vector interactions compatible with plasmid stability under non selective conditions and, thus, to successful exploitation of recombinant organisms in bioreactors for bioremediation.

Recombinant mAW10 strain was immobilized with granular activated carbon (GAC) as supporter matrix for production of ArsR protein. Immobilization could also

decrease the probability for segregational plasmid loss and overgrowth of plasmid-free cells. GAC was found as a support matrix that had good biocompatibility to recombinant mAW10 cells. Cell immobilization was directly dependent on in situ adsorption ability of GAC particles, and it keeps the cells high viability (Fig. 4.12).

5.2 RECOMMENDATIONS

Presently, biological methods are gaining momentum because of their potential in providing a cost-effective technology for metal remediation. The availability of genetic engineering technology provides the possibility of specially tailoring microbial biosorbents with the required selectivity and affinity for As^{3+} .One emerging strategy that is receiving more attention is the use of metal binding recombinant organisms. ArsR proteins are the main metal sequestering molecules used by cells to immobilize arsenite ions, offering selective, high affinity binding sites.

An arsenite water body can be selected and an appropriate microorganism susceptible to such habitat can be genetically engineered to produce arsR protein for bioaccumulation of arsenic, while its chromosomal arsenic resistance mechanism must be deactivated. This recombinant cell can be grown firstly with the selective pressure of antibiotic and later on the selective pressure can be shifted to arsenite and then immobilized in bioreactors for bioremediation of arsenic water.

REFERENCES

1. Rosen, B.P. 2002. "Biochemistry of Arsenic Detoxification." *FEBS Letters*, 529, pp.86-92.

2. Alexander, D.C., Costanzo, M.A., Guzzo, J., Cai, J., Charoensri, N., Diorio, C., and Dubow, M.S. 2000. "Blazing towards the next millennium: Luciferase Fusions to identify genes responsive to Environmental Stress." *Water, Air, and Soil Pollution.* 123, pp. 81-94.

3. Silow, M.B. 2005 "The arsenic Group- Arsenic resistance in Gram-positive bacteria." Department of Cell and Organism Biology, Maria.berggard_Silow@cob.lu.se

4. Trang, P.T.K., Berg, M., Viet, P.H., Mui, N. V., and Van der meer, J.R. 2005. "Bacterial Bioassay for Rapid and Accurate Analysis of Arsenic in Highly Variable Groundwater Samples." *Environ. Sci. Technol.*, 39, pp. 7625-7630.

5. Stolz., J.F., Basu, P., and Oremland, R.S. 2002. "Microbial Transformation of elements: The Case of Arsenic and Selenium." *Ind. Microbiol.*, 5, pp. 201-207

6. Ahmann, D. "Impact of Microorganisms on Arsenic Geochemistry." Division of Environmental Science and Engineering, Colorado School of Mines, Dianne@mines.edu

7. Tauriainen, S., Karp, M., Virta, M.. Recombinant luminescent bacteria for measuring bioavailable arsenite and antimonite. *Appl Environ Microbiol* 63. (1997), 4456-4461.

8. Corbisiera, P., Jib, G., Nuytsa, G., Mergeaya, M. and S. Silver. *Lux*AB Gene Fusions with the Arsenic and Cadmium Resistance Operons of *Staphylococcus aureus* Plasmid pI258.. *FEMS Microbiology Letters*, 110:2,(1993.) pp. 231-238.

9. Petänen, T., and M. Romantschuk.Use of Bioluminescent Bacterial Sensors as an Alternative Method for Measuring Heavy Metals in Soil Extracts.. *Analytica Chimica Acta*, 456:1,(2002) pp. 55-61

10. Ensley, B.D. (1986) *CRC Crit. Rev. Biotechnol.* 4, 263-277.

11. Ollis, D.F., (1982) *Philos. Trans. R. Soc. London Ser.* B 297, 617-629.

12. Nordström, K., Molin, S. and Aagard Hensen, H. (1980*) Plasmid* 4, 215-227.

13. Zabriskie, D.W. and Arcuri, E.J. (1986*) Enzyme Microb. Technol.* 8, 706-717.

14. Meacock, P. and Cohen, S.N (1980) *Cell* 20, 529-542

15. Hershfield, V. Boyer, H., Yanofsky, C., Lovett, M. and Helinski, D. (1974) *Proc. Natl. Acad. Sci.* USA 71, 3455-3459.

16. Godwin, D. and Slater, J.H (1979) *J.Gen. Microbiol.* 111, 201-210.

17. Georgiou, G.(1988) *AIChE J.* 34, 1233-1248.

18. Imanaka, T. and Aiba, S. (1981) *Ann. New York Acad. Sci.* 369, 1-14.

19 Kumar, P.K.R., Maschke, H.E., Friehs, K. and Schügerl. " Strategies for improving plasmid stability in genetically modified bacteria in bioreactors." *Trends in Biotechnology*, 9, 279-284.

20. Schuler, M.L. Kargi, F., *Pearson Education (Singapore)* "Bioprocess Engineering Basic Concepts."

21. Nies, D.H., and Silver, S. "Ion efflux systems involved in bacterial metal resistances." *Journal of Industrial Microbiology*, 14, pp. 186-199.

22. Rosen, B.P. 2002. "Biochemistry of Arsenic Detoxification." *FEBS Letters*, 529, pp.86-92.

23. Mukhopadhyay, R., Rosen, B.P., Phung, L.T., Silver, S. 2002. "Microbial arsenic: from geocycles to genes and enzymes." *FEMS Microbiology Reviews*, 26, pp. 311-325.

24. Turpeinen, R. 2002. "Interactions between metals, microbes and plants- Bioremediation of Arsenic and lead contaminated soils." University of Helsinki, Lahti.

25. Lewis, J.C., Feltus, A., Ensor, C.M., Ramanathan, S., and Daunert, S.1998 "Applications of Reporter Genes." *Analytical Chemistry News and Features*, pp. 579A-585A.

26. Ripp, S., and Sayler, G.S. "Advanced Bioreporter Technologies for Targeted Sensing of Chemical and Biological Agents." http://www.ceb.utk.edu

27. Van der meer, J.R., and Stocker, J. "Bacterial Biosensors to Measure Arsenic in Potable Water." *EAWAG news 56*, pp.12-14.

28. Scott, D.L., Ramanathan, S., Shi, W., Rosen, B.P., and Daunert, S. 1997. "Genetically Engineered Bacteria: Electrochemical Sensing systems for Antimonite and Arsenite." *Anal. Chem.*, 69, pp. 16-20.

29. Tauriainen, S., Virta, M., Chang, W., and Karp, M. 1999. " Measurement of Firefly Luciferase Reporter Gene Activity from Cells and Lysates Using *Escherichia coli* Arsenite and Mercury Sensors." *Analytical Biochemistry*. 272:2, pp. 191-198

30. Ramanathan, S., Shi, W., Rosen, B.P., and Daunert, S. 1997. "Sensing Antimonite and Arsenite at the Subattomole level with Genetically Engineered Bioluminescent Bacteria." *Anal. Chem.* 69, 3380-3384.

31. Ji, G. and Silver, S. 1992. "Regulation and expression of the Arsenic Resistance Operon from *Staphylococcus aureus* Plasmid pI258." *J. Bacteriol.*, 174, pp. 3684-3694.

32. Roberto, F.F., J. Barnes, and D. Bruhn. 2002. "Evaluation of GFP reporter gene construct for Environmental Arsenic Detection." *Talanta*. 58:1, pp. 181-188.

33. Petänen, T., Virta, M., Karp, M., Romantschuk, M., 2001. "Construction and Use Board Host Range Mercury and Arsenite Sensor Plasmids in the Soil Bacterium *Pseudomonas fluorescens* OS8." *Microbial Ecology*, 41, pp. 360-368.

34. Alexander, D.C., Costanzo, M.A., Guzzo, J., Cai, J., Charoensri, N., Diorio, C., and Dubow, M.S. 2000. "Blazing towards the next millennium: Luciferase Fusions to identify genes responsive to Environmental Stress." *Water, Air, and Soil Pollution*. 123, pp. 81-94.

35. Stocker, J., Balluch, D., Gsell, M., Harms, H., Feliciano, J., Daunert, S., Malik, K.A., and Van der meer, J. R. 2003. "Development of a set of simple bacterial biosensors for quantitative and rapid measurements of arsenite and arsenate in potable water." *Environ. Sci. Technol.*, 37, 4743-4750.

37. Miller, J.H.: 'Assay of β-galactosidase' in Experiments in Molecular Genetics. Cold Spring Harbor Laboratories, *Cold Spring Harbor*, N.Y. USA, 1972, pp. 352-355.

36. T. Maniatis, D.F. Fritsch, J. Sambrook, Molecular cloning: A Laboratory Manual, Cold spring Harbor Laboratory, *Cold Spring Harbor*, NY, 1989.

38. http://openwetware.org/wiki/Beta-Galactosidase_Assay_(A_better_Miller)

39. Z. Wang and N.A. Da Silva, Analysis of X-gal plates for Quantitative plasmid stability determinations in *Saccharomyces cerevisiae*. *Biotechnology Techniques* , Volume 7 No. 5 pp. 325-328

40. Neubaur, P. Hofmann, K., Host, O., Mattiasson, B. And Krusckke, P. (1992) Maximizing the expression of a recombinant gene in *Escherichia coli* by manipulation of induction time using lactose of inducer. *Appl. Microbiol. Biotechnol.* 36, 739-744.

41. A. Ramesh, A.K. Panda, B.R. Maiti, Asok Mukhopadhyay, Studies on plasmid stability and LTB production by recombinant Vibrio cholerae in batch and in chemostat cultures: A lesson for optimizing condition for chemical induction. *Journal of Biotechnology* 43(1995) 45-51.

42. Wu, C.M., and Lin, L.Y.: Immobilization of metallothionein as a sensitive biosensor chip for the detection of metal ions by surface plasmon resonance. *Biosensor and Bioelectronics*. 20, 864-871 (2004).

43. Ramanathan, S., Shi, W., Rosen, B.P., and Daunert, S.: Bacteria-based chemiluminescence sensing system using β-galactosidase under the control of the ArsR regulatory protein of the *ars* operon. *Analytica Chimica Acta*. 369, 189-195 (1998).