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Exploitation of Genetic Capacities of Bacteria for Improved Plasmid Stability in Remediation of Arsenic Pollution

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ABSTRACT

In recent time arsenic pollution is a serious threat to the human life. This pollutant is most prevalent in south eastern part of Asia. If not controlled properly it leads to severe diseases like cancer and ultimately death. Till date not too much has been done towards the development of any bioremediation methodology for this highly toxic chemical. There are scanty reports on the development of recombinant bacterial strains which has been used for the bioremediation of arsenic. In this study we have attempted to develop a methodology to improve the stability of arsenic sensitive genes (ars R) in a recombinant bacterial strain. Towards this end, genetically engineered bacterial strain, DH5α, has been transformed with ars R gene which was exposed to the arsenic rich water sample. Our study established that in the presence of arsenic in the medium, this cell is forced to maintain this plasmids and stably expressed in the bioreactors.

Key words: Arsenic Resistance Mechanism, Plasmid Stability, Selection pressure

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INTRODUCTION

As (V) is taken up by the cell via phosphate transporters, and As (III) by aquaglyceroporins. As (V) is reduced to As (III), this is either extruded from the cells or sequestered in intracellular compartments. ars operon consist 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 to extrude arsenite and extrude arsenate by reducing it to arsenite [1]. Inactivating ars operon leads to hypersensitivity to arsenic compounds [2]. In ars operon arsR 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. 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. In our present work we have exploited arsenic resistance mechanism of the bacteria for arsenic bioaccumulation [1].

The metalloregulatory protein ArsR, which offers high affinity and selectivity towards arsenite, is responsible for bioaccumulation of arsenic. Thus ArsR protein offers an inexpensive, high-affinity ligand for arsenic removal from contaminated drinking and ground water [3]. Enhancement in the yield of ArsR requires the analysis of plasmid stability and its consequence on ArsR production. Plasmid stability is one of the most important factors to be considered in the scale-up of a bioprocess involving recombinant organisms. Plasmid DNA production is becoming increasing important not only as gene therapies make their way into clinical trials, in pharmaceutical marketplace but also in bioremediation processes using genetically engineered cells. Through optimization of the growth environment for plasmid producing organisms, improvements can be achieved in biomass yield, plasmid yield and ultimately to the yield of desired protein. The primary goal when designing a bioremediation process using genetically engineered cells is to maximizing yields of supercoiled plasmid. 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 [4]. 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 [4]. 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.

To avoid the risk of spreading antibiotic resistance traits to environmental microbes, use of antibiotic selection is highly discouraged. Hence, we propose a different approach to overcome instability in recombinant organisms employed in bioremediation processes. Here we studied the stability of the pMV132-arsR-ABS plasmid in E.coliDh5α and AW10 strain of E.coli and found that arsenite also exerts pressure on cells to maintain plasmid if and only if plasmid carries genes necessary for its survival in adverse environments [5, 6]. Studies were carried to determine stability of pMV-arsR-ABS in E.coli DH5-α and in AW10 strain of E.coli which has its chromosomal arsenic resistance genes replaced by chloramphenicol resistance genes [4]. Recombinant cell of AW10 strain of E.coli transformed with pMV132-arsR-ABS plasmid was named as mAW10. Thus, the present investigation has been aimed towards increasing the selective pressure by increasing the concentration of arsenite to induce the recombinant cell for better productivity of ArsR for bioaccumulation of arsenic as well as enhanced plasmid stability.

MATERIAL AND METHODS

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.

Strains and plasmid

E.coli DH5-α containing the plasmid pMV132-arsR-ABS was 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* 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 and was lacking arsenic resistance genes instead. So, the bacterial biosensing system mAW10 developed by transforming plasmid pMV132-arsR-ABS in AW10 strain of *E.coli* was resistant to both Ampicillin and chloramphenicol.

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 [6].

Apparatus

Bacterial cells were grown in an Orbital Shaker/ incubator (Metrex Scientific Instruments Pvt. Ltd., India). Chemiluminescence measurements were done in a 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 [9]. Each replica plating experiment was repeated at least thrice and results are presented as the mean of two replicate determinations. Standard deviation of each set of results was less than 10% of the mean value.

Determination of plasmid stability

The plasmid stability was checked by the expression of X-GAL in the presence and absence of antibiotics. The cells were plated in the presence or absence of antibiotics and the numbers of cells were counted. Under the stress of the antibiotics, the cell counts if increased and they respond to the arsenic that could be indicative of the stability of the ars R gene in the bacteris. The same cells were then incubated with increasing concentrations of arsenic and the expression levels of X-GAL were determined. mAW10 were plated on LB agar with 100 µg/mL Ampicillin and X-gal. 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 ºC 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 harbouring 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 were 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 [10]. 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 aseptically 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.

Plasmid stability of mAW10 strain at various arsenite concentrations

Three flasks containing 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 OD600 and after finding them in late exponential growth phase, arsenite was added to each of the flasks. Now, plasmid stability was determined by using the protocol described above.

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 [7,8]. It was observed that after 25 minutes sufficient color was developed, so stop solution (1M Na2CO3) was added and mixed well for mAW10 strain during ONPG test [9,10]. Miller units were calculated as

$$1 \text{ Miller Unit} = \frac{1000 \times \text{OD}_{420}}{\text{OD}_{420} \times \text{Volume (mL)} \times 25(\text{t, min})}$$

RESULTS AND DISCUSSIONS

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. The resulting cells become non-productive. The plasmid pMV132-arsR-ABS was specially designed for reduced basal expression [5]. 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 [5]. Minimization of basal transcription is especially important when the expression target introduce a cellular stress situation and thereby selects for plasmid loss.

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 [7,8]. 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 (Fig. 1). 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 [11-12].

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 (Fig. 2). Here, dose response curve in terms of Miller Units quantifying beta - galactosidase activity, given in the following equation has been drawn for various arsenite concentration after the bacteria mAW10 were incubated with sodium arsenite for 30 minutes. The x-axis reflects the arsenite concentration in the sample. Data are the mean standard deviation of three independent experiments. 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 [13-17]. It has been found that high stability was achieved at high concentrations of arsenite (Fig. 3).

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. Whereas, 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.

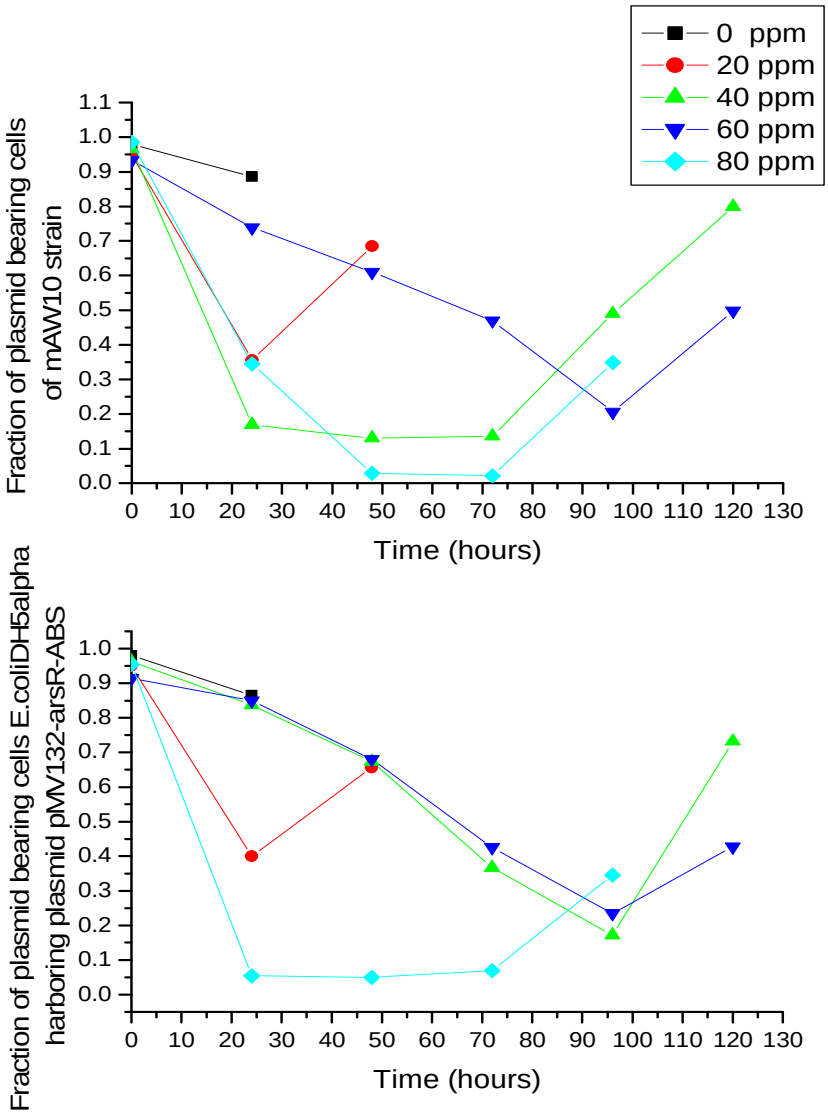

Fig. 1 Comparison of plasmid stability of mAw10 and *E.coliDh5α* strain harboring the plasmid pMV132-arsR-ABS at three different phases of growth with time course when induced by various arsenite concentration during inoculation

CONCLUSION

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 As3+. One emerging strategy that is receiving more attention is the use of metal binding recombinant organisms [18, 19, 20]. ArsR proteins are the main metal sequestering molecules used by cells to immobilize arsenite ions, offering selective, high affinity binding sites [21].

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.

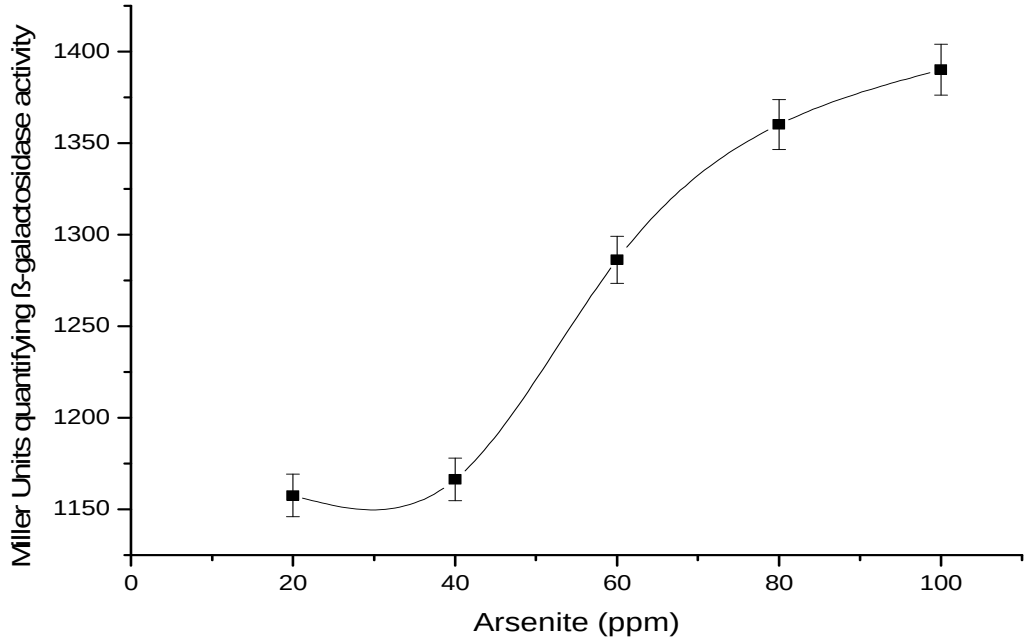

Fig. 2 Dose-response curve for various arsenite concentration, expressed in terms of Miller Units quantifying β - galactosidase activity

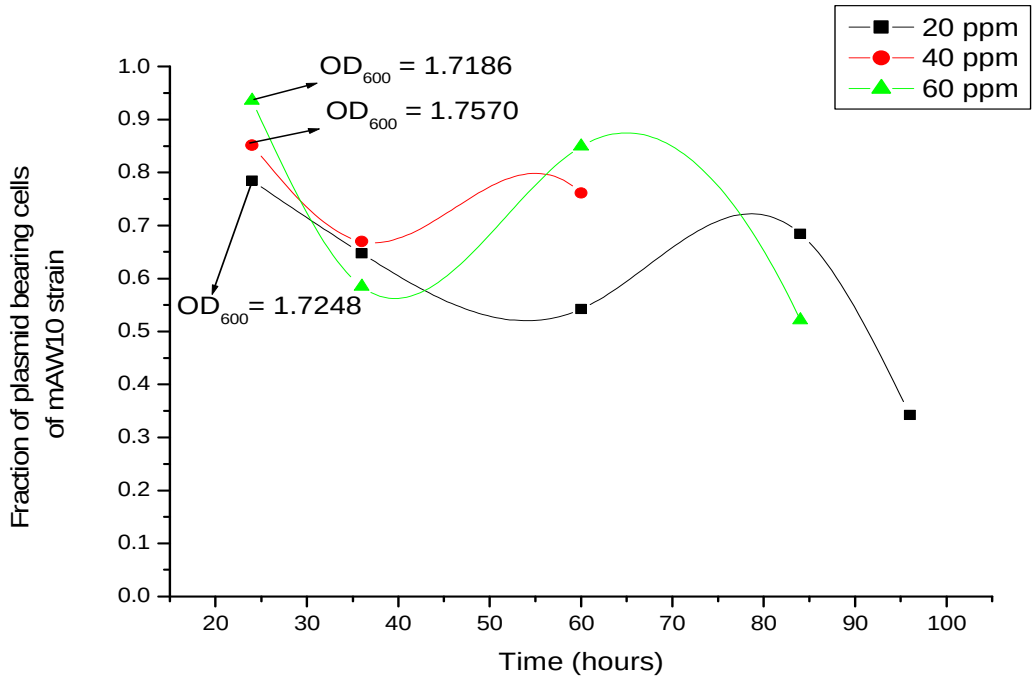

Fig. 3 Time trajectory of plasmid fraction in mAW10 strain induced at late exponential phase at various arsenite concentrations

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.

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