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Isolation of bacteria with arsenite mobilization capacity from culture and water sample from arsenic area of Bihar, India.

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Abstract: *Toxicity of heavy elements like arsenic has become a global concern owing to the increasing contamination of soil, water and crops in many regions of the world including India. Arsenic, known to induce skin lesions, cancers, immune-suppression and other symptoms reported to cause testicular toxicity leading to cancer in arsenic-treated male mice in our laboratory. For the treatment perspective, on the other hand, we have focused on the growing interest in the studies of probiotics development for the arsenic-toxicity disorders. Microorganisms have been shown to play important roles in the biochemical cycle and arsenic-speciation by different mechanisms (transformation/metabolism by oxidation, reduction or methylation etc.) to cope with raised concentration of arsenic exposure. In the present study, with an aim to get bacteria capable of degrading arsenic, three strains of E. coli (TG1, DH5α, BL21);one strain of B. subtilis (MTCC-4445) were used relating to previous literature and also, two field-water isolates Fwi-C2 & Fwi-C3 were taken from Arsenic affected area; and their capability for potential arsenic immobilisation were experimented in nutrient (agar and broth) media supplemented with sodium arsenite at various concentrations (50, 100, 200, 400, 500, 700 µg/ml for each). The standard protocol and conditions for culture of these bacteria were followed. After the measurements of bacterial cells suspensions and counting the viable cells; the remaining concentrations of arsenic (after the growth of bacteria up to 96 hours incubation at standard conditions) in the culture fluids were estimated by arsenic kit method (Merck, MQuant™) and graphite furnace atomic absorption spectroscopy (GF-AAS). There was observed a significant growth of the test strains of bacteria at even 400 µg/ml added with Sodium Arsenite (by E. coli*DH5α, BL21*; and Fwi-C3) and also at 500 µg/ml (E. coli TG1) and B. subtilis (MTCC-4445). But there was found no degradation of arsenic supplemented in the culture media; which indicate the development of bacterial tolerance to Arsenic (As+3) by the microbial strains tested in the study.*

Key words*: E. coli, B. subtilis, Field-water isolates, high Arsenic concentration; Arsenic Kit test method; GF-AAS.*

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I. Introduction

Arsenic is a toxic metalloid that commonly remains in the environment in trivalent and pentavalent forms; releasing into the environment either by natural phenomena (weathering, volcanic activity)or by anthropogenic activities [1,2]. Arsenic toxicity has become a significant concern world-wide due to the increasing contamination of soil, water and crops and also such toxicity depends on various factors such as physical state, particle size, adsorption rate, solubility etc. Arsenic is known to induce skin lesions, cancers, immunosuppression and other symptoms [5-6]. Currently available physico-chemical methods used for removing arsenic from contaminated environment have many disadvantages because of the vast polluted area, high cost, generation of secondary contaminants etc. Therefore, development of technologies to reduce costs involving biotechnological approaches for treatment of contaminated environment has stimulated serious interest in studies of the bioremediation of toxic metals like Arsenic.

Microorganisms play important roles in the biochemical cycle and arsenic-speciation [11]. Bacteria have evolved a number of mechanisms to cope with, or even benefit from, arsenic exposure [9]. A number of microbes have been isolated that use arsenic in their metabolism, either using arsenate as a terminal electron acceptor in anaerobic respiration [1,20], or as a means of generating energy through chemoautotrophic arsenite oxidation [19].Bacteria induced arsenic transformation involves oxidation, reduction or methylation to overcome the toxic effects and survive in arsenic-rich environment. Earlier work suggested the involvement of arr genes, ars operon and aox genes in arsenic resistance and metabolizing systems in bacteria [11]. In Escherichia coli, an ArsA-ArsB complex functions as a primary arsenite pump [12].

II. Background

There found high contents of Arsenic (up to 300-500 ppb) present in water and soil in Bihar; particularly in the Gangetic region which was quite higher than the WHO limits (10 ppb) in drinking water. Previously, our group [2] reported about testicular toxicity in adult Swiss Albino male mice due to Arsenic (2mg/kg/day) doses administered till 4-6 weeks. In that study, damaged seminiferous epithelial layer and degeneration of spermatogonia with increased visibility of interstitial space in testes of mice under Arsenic treatment in comparison to normal (control) mice were shown. However, for the remedial benefits, studies on screening and development of probiotics were conceived (*figure-1*) and process was implemented to find microorganisms with potential for Arsenic degradation. In a pilot study, Arsenic affected field samples were taken and microbes were isolated on suitable media supplemented with arsenic and finally their potential to degrade arsenic was experimented at different concentrations. In addition relating to previous literature, few strains of *E.coli* and *B. subtilis* were in parallel taken for this study as similar bacteria play a beneficial role in intestine being present in the normal microbial flora. In such experiments, the growth parameters and arsenic degradation potential of the bacterial strains were evaluated.

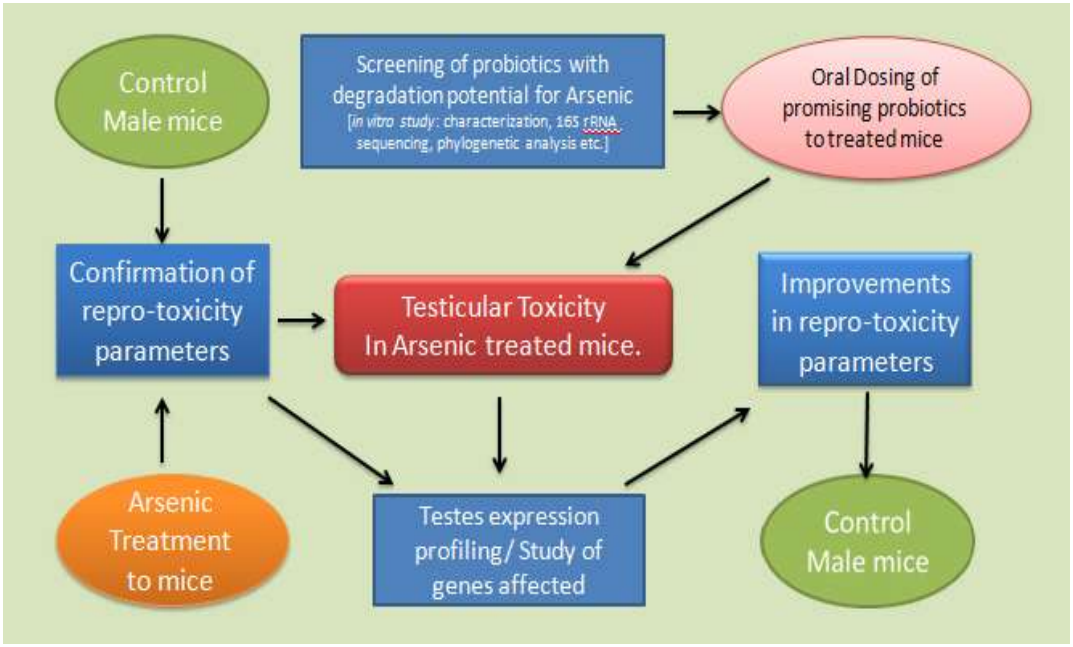

Figure 1: Schematic theme of study-Arsenic induced testicular toxicity in mice & probiotics screening. Testicular toxicity in arsenic treated mice confirmed by repro-toxicity parameters and gene expression profiling (Microarray). The improvements in the repro-toxicity parameters (conferred by probiotics candidates with arsenic degradation potential) aimed to be evaluated for remedial assessment.

III. Materials And Methods

3.1. Culture of Bacterial Strains.

5ml of NB (Nutrient Broth) media was freshly prepared in a test tube in which inoculum of 5 µl of bacteria [*E. coli (DH5α, BL21 & TG1)* and *Bacillus subtilis*] cells were grown aseptically (from 20% Glycerol stock of bacteria) at 37^0 C, 150 rpm in a shaking incubator for 16 hours (as described in *Cappuccino & Sherman, 2007*). The strain of *B. subtilis* was purchased from MTCC-IMTECH Chandigarh while the *E. Coli* strains were received as gifts; [*E. coli TG1* from Ms.Manpreet Sodi, IISER-Mohali; and *E. coli (DH5α and BL21)* from Dr. Amit Roy, NIPER-Hajipur]. When the optical density (O.D) of the culture reached up to 0.3 to 0.4, then further 10 µl of freshly grown cells were sub-cultured in another 5ml of NB media prepared aseptically.On the other hand, Nutrient Agar (NA) plates were prepared with desirable concentrations of antibiotics (Chloramphenicol; CAM) or with Arsenic (As^{3+}) from Sodium arsenite (LobaChemie). All these were prepared aseptically in the laminar hood according to the calculations tending to the working concentrations as required in the experiments. After the solidification (preparation) of NA plates, 70 µl of the exponentially grown cells were spread with respect to the estimate of working concentrations of substances (CAM/As^{3+}; as calculated beforehand) in the laminar hood avoiding all sorts of contamination chances. After plating of the bacteria, the NA plates were properly kept in the incubator at 37^0 C for 16 to 20 hours until the colonies appear as per the standard protocols.

3.2. *Field sampling of water.*

From arsenic affected area Vaishali, Bihar (85. 375 ^{0}E, 25.625 ^{0}N) several drinking water samples were taken in sterile plastic vials and estimated for the concentration of Arsenic occurring in them. The water samples (≥50 ppb of Arsenic) were brought to the laboratory and stored immediately at 4-8 ^{0}C. Next day, the water samples were filtered through Whatmaan filter paper and diluted serially up to 10^6 times which were further isolated on freshly prepared NA plates with suitable control. Then re-streaking was done to further get pure Field-water isolates (i.e. Fwi-C2 & Fwi-C3) that were experimented on NA media supplemented with different concentrations (0,50,100,200,500,700 µg/ml) of Sodium Arsenite and separately with Chloramphenicol.

3.3. Measurement of optical density (Absorbance) of bacterial cells.

The absorbance of bacterial cells [*E. coli (DH5α, BL21, TG1) and B. subtilis*] grown separately (without and with arsenic treatment *viz.* 100, 300, 600 µg/ml) in LB mediaat 37 ^{0}C, 150 rpm in shaking incubator; were measured by **Color Eye (IBL)** colorimeter at three different wavelengths [540 nm, 580 nm and 610 nm] after incubations of 16, 36, 72 hours. The readings of absorbance at 610 nm wave length were recorded and tabulated by taking only LB media as blank standard; (Absorbance readings at 540 nm, 580 nm found not very relevant, hence were not shown in the data).

3.4. Counting of bacterial cells using Trypan Blue Exclusion Method.

From the previously grown bacterial culture, 7µl volume of cells were taken in a 1.5 ml micro-centrifuge tube (Abdos); and to it added 7µl of Trypan blue (0.4%; w/v) solution. It was mixed by gentle pipetting. From this mixture, 12 µl was loaded to the Haemo-cytometer (Neubauer Improved Superior Marienfeld). By the usual method of Haemocytometry, the total number of bacterial cells per 1 ml LB media; were counted under microscopy (40X) and multiplied with the dilution factor 2; (here as culture: Trypan blue taken in 1:1) by applying the standard formula.

3.5. Estimation of concentration in bacterial culture supplemented with arsenic.

3.5.1 By Arsenic Field Test kit method: (Merck, MQuant^{TM})

Materials and reagents of Test Kit:Field Test Kit (Merck, Germany) contained two test bottles, one red spoon, one green spoon, reagent 1st (As-1), Reagent 2nd (As-2), Reagent 3rd (As-3) and arsenic test strips.

Method for detection of arsenic using test kit: The sample to be estimated was filled in the reaction bottle up to 60ml mark. Then two drops of **reagent As-1**, were added and swirled well holding the bottle vertically. **Reagent As-2** was then added [1 level] with the Red dosing spoon, and swirled well till the reagent was completely dissolved. Subsequently, **ReagentAs-3** was added [1 level] with the Green dosing spoon, and immediately the reaction bottle was screw-capped to avoid any gas exchange. Soon after this, the test strip was inserted as far as it could be put down in the reaction bottle, through the hole of the black-colored cap. Much care was taken so that test strip could not touch the liquid sample in the reaction bottle. The reaction bottle was kept as such for 20 min, swirling two or three times during the period. The reaction zone color (which might be changed after the reaction time) was compared with the reference display of standard color bands of Arsenic kit [Min. conc. 0.0 mg/l to Max. conc. 0.5 mg/l].The samples containing more conc. of this maximum range, were suitably diluted by distilled water; and after actual measurement of arsenic conc., the dilution factor was multiplied with the estimated value.

3.5.2. By GF-AAS (Graphite Furnace Atomic Absorption Spectroscopy) Method.

***Reagents of AAS*:** HCl, $HClO_4$, Conc. HNO3 and Distilled water, Purge gas [Mixture of Hydrogen (5%) and Argon (95%)]. The chemical matrix modifiers, for the arsenic determination with the GF-AAS analytical technique, were Ni, Pd and a Pd–Mg mixture*: i.e*. the most commonly mentioned in the literature [16].

Method of GF-AAS sampling: It was performed as per the standard protocol with slight modifications. Briefly, the culture fluid taken in a 100ml conical flask was put in digestion with 100% HNO_3overnight. Then second phase digestion was set with HNO3 and HClO4 (1:1) for 3-4 hours over hot plate following further processing (making up final volume, sample loading, estimation etc.) were performed in Atomic Absorption Spectrophotometer (AAS), Perkin Elmer, model number PinAAde900T; at Mahavir Cancer Sansthan & Research Institute, Patna, India.

3.6. Pre-processing of bacterial culture fluid before Arsenic Estimation by Kit Method.

As the Arsenic kit method could estimate As^{+3} or As^{+5} only in a very small range (0 to 0.5 mg/l), the culture fluids initially added with SodiumArsenite; was diluted by distilled water in such a manner that the

working concentration of the test culture fluid would fall in this range which could be displayed with one of the appropriate colour-strip. The bacterial culture was centrifuged at 6000 rpm and the supernatant was subjected to suitable dilution. For example, to test at 0.25 mg/l (equivalent to 0.25 µg/ml), a bacterial culture fluid of 300 µg/ml was diluted 1200 times with distilled water; and finally the result was compared with the reaction strip colour-display provided with the kit. Apart from the supernatant, the bacterial pellet was also lysed with lysozyme (100µg/ml) and 10% SDS (w/v). The cell lysates were also then filtered and the soup was suitably diluted as described above to check if bacteria could consume any traces of Arsenic.

3.7. Statistical analysis.

The graphical analysis/drawing was done using MS Excel 2013 or Graph Pad Prism 5.0. Statistical analyses were performed using SPSS software package 16.0.

IV. Results And Discussion.

The bacterial strains of *E. coli* (TG1, DH5α , BL21) and *B. subtilis* (MTCC4445) were mainly cultured both in NB and NA media supplemented with different concentrations (0,100,200,500,700 µg/ml) of Sodium Arsenite (As+3) as shown in the *table-1*. In addition, two field isolates were also included to check their growth potential in arsenic supplemented media. The experiments were carried as per standard protocol with taking antibiotics (CAM) supplemented media as control plate. It was observed that all the strains show significant growth potential up to 500 µg/ml of added Arsenic in both NA & NB media.

Bacterial strains	Media only [NA&NB]	**100$** [Arsenic, As^{+3}]	**200$** [Arsenic, As^{+3}]	**500$** [Arsenic, As^{+3}]	**700$** [Arsenic, As^{+3}]	**35$** [Chloram phenicol]
E. coli DH5α	**LG**	**+++**	**++**	**+**	**NG**	**NG**
E. coli BL21	**LG**	**+++**	**+**	**+**	**NG**	**NG**
E. coli TG1	**LG**	**+++**	**+++**	**+**	**NG**	**NG**
B. subtilis (MTCC:4445)	**LG**	**+++**	**+++**	**++**	**NG**	**NG**
Fwi-C2	**LG**	**+**	**+**	**NG**	**NG**	**NG**
Fwi-C3	**LG**	**+++**	**++**	**++**	**NG**	**NG**

***Table-1*:** Bacterial growth pattern in presence of Arsenic (As^{+3})on Nutrient Broth (NB) & Nutrient Agar (NA) media.

LG: Luxuriant growth, **+++**: very high growth, **++:** well growth, **+:** significant growth. **NG:** No growth**.**
[Working Conc: ($ = µg/ml). The result shown here is the mean of triplicates of the experiments ($p<0.05$)].

In presence of added Arsenic in media, E.coli, TG1 was found to have better growth potential (i.e. very high growth at 100 µg/ml ; significant growth at 500 µg/ml) than other two E.coli DH5α (i.e. well growth at 100 µg/ml ; significant growth at 500 µg/ml) and E.coli BL21 (i.e. significant growth at both 100 &500 µg/ml). The observations were based mainly on the colony morphology of the bacterial plates. The field-water isolate Fwi-C3 was found to have better growth potential than another field-water isolate Fwi-C2. No growth was observed at 700 µg/ml of arsenic concentration, neither at Chloramphenicol added culture media (control).

The absorbance (optical density) readings were measured (in different concentrations of Arsenic) for each of the strains of bacteria at 610 nm wavelength by Colorimeter (IBL) as shown in *figure-2*. There was found higher OD-values of bacteria (in NB media with 300 µg/mlAs^{+3}) than absorbance profiling at 700 µg/ml of arsenic added in the media.

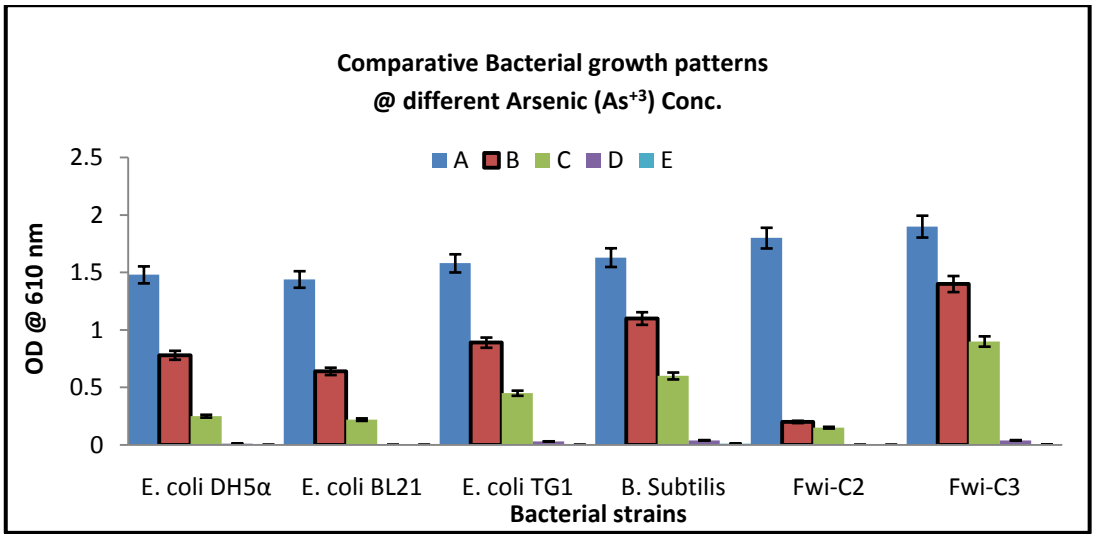

***Figure-2*:** Growth pattern of different bacterial strains in culture media added with different conc. of arsenic (As^{+3}) at standard conditions.
[Conc. $= µg/ml; **A:** 0$ As^{+3}; **B**: 100$ As^{+3} ; **C**:300$ As^{+3}; **D:** 700$ As^{+3} ; **E**: 35$ Cam].
The result shown here is the mean of triplicates of the experiments ($p<0.05$)].

There was highest turbidity of bacterial culture recorded in only NB media with no added arsenic and no reading was observed in NB culture media with added antibiotics Chloramphenicol (35µg/ml).

Among most of the test strains, there was found almost significant growth at 200-300 µg/ml of Arsenic added in the media. Hence the comparative profiling was done (at 250µg/ml of Arsenic concentration added in media) with all the bacterial strains under screening as shown in figure-3.

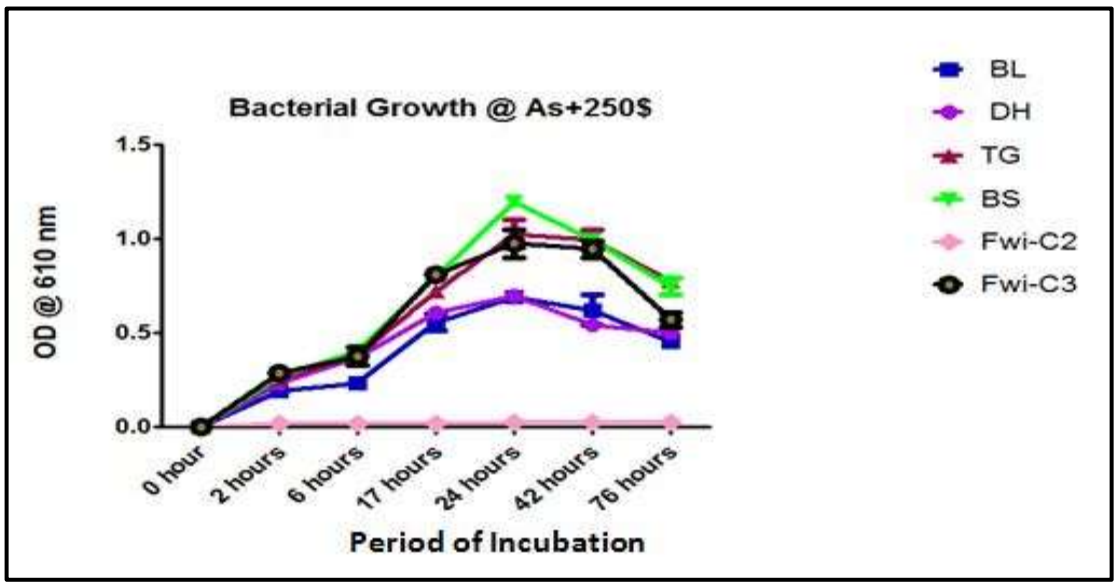

Figure-3: Absorbance at 610 nm of different bacteria growth in culture broth added with 250 µg/ml Arsenic after incubation [0, 2,6,17,24,42,76 hrs]

At 370C, 150 rpm in rotary shaker**. $:**µg/ml conc.; **BL**: *E.coli* BL21;**DH**: *E.coli* DH5α;**TG**: *E. coli* TG1;**BS**: *B. subtilis* (MTCC4445);
Fwi-C2&**Fwi-C2**: Field-water isolates C2&C3. The result shown here is the mean of triplicates of the experiments ($p<0.05$)].
B. subtilis was observed to have better growth potential among other bacterial strains (*E. coli* TG1, DH5α, BL21) at the same concentration 250 µg/ml in normal conditions as described in material and methods. The field-water isolate Fwi-C3 was found to be growing better in comparison to other field-water isolate Fwi-C2.

Although some strains shown significant growth at high As+3 concentration, the Arsenic degradation potential was examined by Kit method as described in Materials and Methods. The process was pre-standardised with suitable dilutions of culture fluid before estimation of Arsenic in bacterial culture fluids.

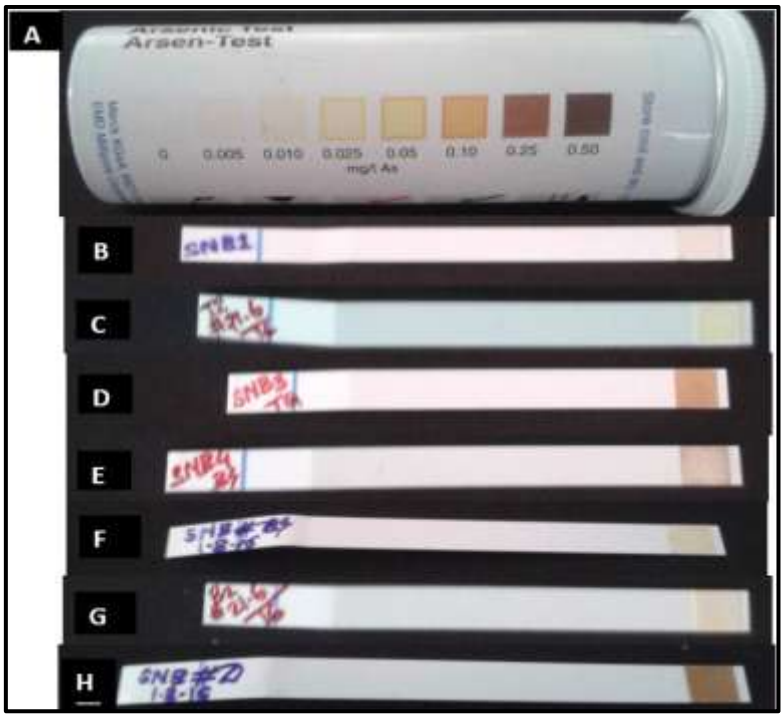

Figure-4: Photographs of the test strips of Arsenic Kit.(A) The reference display of standard colors (eight) as given in the Arsenic kit method* (max. range up to 0.5 mg/l). (B) White color indicates 0.00 mg/l arsenic in LB media without arsenic treatment. (C) Faint yellow color in the reaction zone of the strip shows 0.01 mg/l arsenic in LB media having *E. coli TG1* with added arsenic. (D) Brown color in the reaction zone of the strip shows 0.05 mg/l arsenic in LB media having *E. coli TG1* with added arsenic. (E) Yellowish orange colorin the reaction zone of the strip shows 0.05 mg/l arsenic in LB media having *Bacillus subtilis* with added arsenic.(F) Yellow color in the reaction zone of the strip shows 0.025 mg/l arsenic in LB media having *Bacillus subtilis* with added arsenic. (G) Faint yellow color in the reaction zone of the strip shows 0.05 mg/l arsenic in LB media *E. coli BL21* with added arsenic(H) Dark grey color in the reaction zone of the strip shows 0.5 mg/l arsenic in LB media having *E. coli DH5α* with added arsenic. *The displayed color zone of the test strips were standardized, the exact concentrations of arsenic in the samples are dependent on the appropriate dilution factors.

The initial concentration of Arsenic in the culture supernatant was found as the final concentration which was back calculated by multiplying the dilution factor (DF) with the observed As^{+3} concentration of the culture fluid (Table-2). All the strains of bacteria showed significant growth at different Arsenic concentrations as supplemented in their respective culture media. By using Trypan blue dye, the live bacterial cells after incubation (16 hours and 72 hours) were counted on Haemocytometer as described in materials and method. The increasing OD reading was observed due to increasing bacterial cells in their corresponding media.

Microbial Strains	Conc.**i** **(As^{+3}) (µg/ml)**	D.F.	Conc.**w** **(As^{+3}) (µg/ml)**	Conc.**o** **(As^{+3}) (µg/ml)**	Conc.**f** **(As^{+3}) (µg/ml)**	**(As^{+3})**Degradation /Conc. Reduction as present initially	No. of viable cells/ml	
							After 16hrs	After 72hrs
E. coli TG1	300	1200	0.25	0.25	300	No degradation observed as Conc.**i** = Conc.**f**	1.1×10^6	2.7×10^6
E. coli BL21	100	400	0.25	0.25	100		1.3×10^6	2.4×10^6
E. coli DH5α	250	1000	0.25	0.25	250		1.4×10^6	2.8×10^6
B. subtilis (MTCC-4445)	300	1200	0.25	0.25	300		1.2×10^6	2.4×10^6
Fwi-C2	50	200	0.25	0.25	50		1.7×10^4	1.1×10^5
Fwi-C3	100	400	0.25	0.25	100		1.2×10^6	2.7×10^6

***Table2*:** Bacterial culture fluids profiling of different strains of bacteria in their respective culture fluids by Arsenic kit-method after taking suitable dilutions. The total number of cells per 1 ml of culture counted by using 0.4% (w/v) Trypan blue in haemocytometer; after 16 and 72 hrs of incubation [16, 36, 72 hrs] at 370C, 150 rpm

in rotary shaker. Conc.i: Initial concentration; Conc.w: Working concentration; Conc.o: Observed concentration; Conc.f: Final concentration; D.F.: Dilution factor

In the experiments with Sodium arsenite, there was observed a significant growth of the test strains of bacteria at even 400 µg/ml added As^{+3} *(E. coli DH5α, BL21)* and also at 500 µg/ml [(*E. coli TG1*) and *B. subtilis* (MTCC-4445)].To get confirmation about their growth potential, the corresponding experimental conditions (as done in NA plates) were performed also in Nutrient Broth (NB) test tubes. While measuring the absorbance of the culture tubes at 610nm wave length (without and with arsenic treatment *viz.* 100, 300, 600 µg/ml) by Color Eye (IBL), the turbidity (O.D.) readings were found to be increasing with the increasing durations of incubation (16, 36, 72 hours) at 37^0 C with 150 rpm in rotary shaker. To check whether any reduction in the initial working concentration of added Arsenic, Kit-based method was pre-standardized and used which was found similar in trends for the Arsenic estimation by GF-AAS. After testing the trace concentrations of arsenic in the bacterial culture tubes (by Arsenic kit method; Merck, MQuant^{TM}), there were found no degradation of added arsenic in spite of 96 hours of incubation in suitable conditions as mentioned earlier. The bacterial cells were also found to be increasing (*from 1.2 × 10^6 to 2.7 × 10^8 C.F.U.;approx.*) when counted at different incubation intervals by using 0.4%Trypan Blue in Haemocytometeras per their growth in suitable culture conditions.

V. Conclusion

There was observed good growth of the test strains of bacteria at a significantly high concentration of arsenic about 400 µg/ml added As^{+3} (*E. coli DH5α and BL21*) and also at 500 µg/ml (*E. coli TG1*). *B. subtilis* also found to be growing well at around 500 µg/ml of added arsenic in bacterial broth culture. There was only found Bacterial resistance to As^{+3} [no degradation of arsenic (into its metabolites)] so far in this study. Field isolates i.e. Fwi-C2 and Fwi-C3 (taken from the arsenic affected area in Bihar) were seen to grow at also high As+3 concentration, but in no case there was noted any reduction of initial concentration of added Arsenic in spite of bacterial growth till long durations up to 96 hours.There was found Arsenic tolerance observed in the microbial strains.

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