Summary
Summary (Salient Features)

- Surface water samples were collected from different sites of Goa, which include different beaches along the Goan coastline, estuaries, sewage water and well water from residential areas; and the physicochemical characters of water samples were determined as temperature 28 to 34°C, pH 5.1-8.5, salinity 17.79-34‰, nitrate 4.0-176 µg/L, nitrite 0.15-48.6 µg/L and dissolved oxygen 1.75-7.7 cm³/dm³.

- Viable counts of bacteria in these water samples revealed that 0.5-1% of natural bacterial population can grow in presence of 10mM arsenic (Arsenate), whereas, 0.01-0.2% of these bacteria are able to survive even 50 mM Sodium arsenate.

- The six potent arsenate resistant bacterial strains were selected after continuous subculturing in MSM agar in presence of Sodium arsenate (50mM) and were designated as SI9, BL9, MPT4, Maj4, Man1 and Man2.

- Using 16s rDNA sequencing and NCBI-BLAST these six bacterial strains have been identified as Vibrio sp. NAP-4 (strain SI9), Vibrio campbelli (strain BL9), Aeromonas punctata (strain MPT4), Vibrio sp. CJ11050 (strain Maj4), Pseudomonas anguilliseptica (strain Man1) and Vibrio sp. (strain Man2).

- We have for the first time reported the presence of arsenic resistance in the genus Vibrio which was till now not reported although the arsenic resistance in bacteria is known for the past 50 years or so.

- All these six isolates were found to grow well in presence of upto 150 mM sodium arsenate in MSM broth as well as agar (also in other media like nutrient agar, zobell marine agar and glycerol based marine agar, respectively).

- Interestingly, cell growth in presence of arsenate was enhanced in three strains viz. SI9, Maj4, and BL9.

- All the six isolates selected were resistant to sodium arsenite also upto atleast 2 mM whereas, the strain MPT4 could tolerate upto 6 mM arsenite in MSM broth.

- The optimum temperature, pH and salinity for growth of these six arsenate resistant bacterial isolates were 28±2°C, 6.8-8.0 and 1.5% (15%), respectively.
Glucose was selected as the best carbon source and the sea water based minimal medium with limiting phosphate (SBMLP) medium was designed using this as the sole source of carbon and 65 μM phosphate (limiting concentration) for the uptake studies wherein, presence of phosphate causes hindrance during arsenate estimation by the Molybdenum blue method.

All the six arsenate resistant strains were resistant to some or the other common antibiotics, viz. ampicillin, tetracycline, Nalidixic acid etc. including some other antibiotics like Co-trimoxazole and Cephalexin.

Five out of these six strains were found to contain the arsC (arsenate reductase) gene, the most 3' gene of the ars operon (in most cases) responsible for the first step of arsenate detoxification. The Aeromonas punctata strain MPT4 was PCR negative for arsC gene indicating the presence of some other tolerance mechanism.

We are the first to successfully amplify and sequence the arsC gene from the members of genus Vibrio. These Vibrios seemingly have evolved their arsenic resistance system with the E.coli ars operon and is more closer to the chromosomal ars operon of E.coli W3110.

Four of these 5 PCR positive strains viz., BL9, Maj4, Man1 and Man2 have the arsC gene on the chromosomal genome, whereas, the gene was present on the plasmid of one of these isolates, Vibrio sp. SI9. The size of the amplicon was 353-356 bp in all 5 strains, as expected and the sequences were monophyletic in origin with E.coli W3110 showing 98-99% homology with the chromosomal arsC gene.

The five bacterial strains which were PCR positive did not show any intracellular accumulation of arsenic with respect to time (0-24h), as revealed by HG-AAS experiments, which further strengthens the presence of an efflux system coded by the ars operon genes. The strain MPT4 which is PCR negative, showed bioaccumulation of arsenic as evident from subsequently increasing levels of arsenic w.r.t. time.

SI9 showed 56% and 66.6% uptake after 24h and 48h respectively. Whereas, 46.6% and 50% uptake was noticed in case of the isolate Maj4 after 24 h and 48h of incubation. Significant uptake of arsenate was observed in case of SI9 (51.6%) and Maj4 (68.5%) after 20h (max. growth) in SBMLP medium also.

There was no increase seen in the arsenate uptake levels after 20 h in the phosphate limiting medium because the cultures lose their viability in nutrient deprived conditions.
The SDS-PAGE profile of total protein of the isolates did not reveal any induced proteins in presence of arsenate except for *Aeromonas* sp. MPT4 which showed two induced bands of 46 KDa and 28 KDa.

On the basis of higher tolerance limits for arsenate, enhanced growth rate in presence of arsenate and higher uptake rate for arsenate and presence of *arsC* gene on the plasmid, the isolate SI 9 was selected for cloning of putative *ars* operon.

The reduction of arsenate to arsenite was detected in case of *Vibrio* sp. (strain Maj4) on TLC. The arsenate present in the medium was getting depleted w.r.t. time as evident from the uptake experiments by Molybdenum blue method.

An attempt was made to clone the plasmid borne putative *ars* operon of plasmid pSI9 by means of genome walking technique. The blunt ended fragments of pSI9 generated by Dra I, Eco RV, Pvu II and Stu I were ligated to adaptors and amplification was carried out using adaptor specific forward and *arsC* gene specific reverse primer.

Although many PCR amplified DNA fragments were obtained by the above method, the fragment of highest molecular weight (2.5 Kb) amplified from the Eco RV genome walker library was selected to clone into the vector pSK+ using *E.coli* DH5a host. The transformants were able to grow in the presence of 10mM sodium arsenite.

Upon digestion of the recombinant plasmid (pSK+ with the 2.5 Kb insert) with *Pvu* II (whose recognition sites are closely flanking both the sides the Eco RV site into which the *ars* operon fragment of 2.5 Kb was cloned) gave 4 bands of which the larger fragment was ~2.4 Kb. The vector without the insert did not show this band.

Therefore we can conclude that this 2.5 Kb Eco RV fragment contains partial *ars* operon which is capable of conferring enhanced resistance levels to the host DH5a, which is otherwise resistant to only low levels of inorganic arsenic.
Future Prospects

Arsenic contamination in the ground water as well as surface water bodies is a global problem and hence novel cheap and accurate methods are required for monitoring its presence in environmental samples and bioremediation. As five of our bacterial isolates are showing the presence of ars operon genes, the presence of a highly inducible arsenate/arsenite inducible promoter is expected in one or more of these bacteria. The strongly inducible promoter can be fished out by means of Genome Walking technique. We have already made an effort in this direction by cloning the 2.5 Kb region upstream to arsC gene of SI9 plasmid. The promoter can be reached by performing another walk using a primer designed based on the extreme 5' region (few 100 nucleotides) of this fragment. This promoter can be made use of in construction of a whole cell bacterial biosensor using E.coli JM 109 and the efficiency of the inducible promoter be enhanced by means of site directed mutagenesis. We suggest that a sincere attempt must be made to amplify the other probable genes of the ars operon like arsB, arsA, arsD, arsR, arsH, arsO, arsT, arsM etc. by means of PCR and different types of ars operons present in these isolates should be explored. The presence of any new gene(s) in the operon can be detected by employing the genome walking and sequencing techniques in combination with homology search in the available nucleotide databases like NCBI. One of our achievements is the isolation of an Aeromonas strain which can hyperaccumulate arsenate and can be used for bioremediation. Further characterization of this strain (MPT4) is required in order to elucidate the exact biochemical and molecular mechanism involved in hyper-accumulation of arsenate. The gene(s) responsible for hyper-accumulation can be cloned into any marine algae or higher plants and can be used for reclamation of contaminated sites.

As multiple heavy metal resistances are well known in bacteria, the cross tolerance of these arsenate resistant strains must be studied towards other heavy metals also e.g. Mercury, Cadmium, Copper, Nickel, Zinc, Iron, Aluminium etc.
Some *in-situ* hyper-accumulation as well as arsenate reduction, uptake and biotransformation studies must be performed using water from the arsenic contaminated sites to prepare the Minimal medium for growing these bacteria.

Therefore, we suggest that much in-depth research is required to understand the complete mechanism and sequence of biochemical reactions involved in arsenate detoxification and biotransformation. This study would ultimately enable us to check the potentials of these bacterial strains for bioremediation of arsenic contaminated marine, estuarine and freshwater sites.