

Deciphering the Multi-Dimensional Abilities of Indigenous Bacteria *Enterobacter Cloacae* Isolated from Arsenic Contaminated Industrial Sites

Reeta Bhati

Amity Institute of Microbial Technology

Smitha Mony Sreedharan

Amity Institute of Microbial Technology

Rajni Singh (✉ rsingh3@amity.edu)

Amity Institute of Microbial Technology <https://orcid.org/0000-0002-2862-7011>

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Abstract

Arsenic (As) is a quintessential toxic metalloid and it has been classified as Group 1 human carcinogen. The evolution of arsenic defense mechanisms due to the omnipresent nature of arsenic has resulted in its alteration to less toxic forms. The present study deals with the isolation of arsenic remediating microbial strains from soil samples and their integration into bioremediation strategy. From the metal contaminated site, 118 different bacterial strains were isolated from heavy metal contaminated site. Twenty-five strains were tolerant to arsenic and one bacterial strain *Enterobacter cloacae* (RSC3) demonstrated maximum growth at high concentration of arsenate (6000ppm). The cell growth kinetics of RSC3 revealed the specific growth rate (μ) to be 0.55 h^{-1} . The bacteria hosts *arsC* gene in the genome involved in the reduction of arsenate to arsenite. AAS, SEM, TEM and EDX studies confirmed the arsenate transportation and efflux of arsenic by the bacteria. Furthermore, the strain showed multi-resistance to other heavy metals like zinc, cadmium, selenium and nickel and several antibiotics indicating its application for facilitating bioremediation of toxic metal contaminated sites.

Introduction

Last few decades have seen tremendous rise in the environmental pollution in all forms. The contamination of soil and water resources by toxic metals/heavy metals is a major cause of health and environmental concern worldwide. The heavy metals include cadmium, arsenic, mercury, zinc, copper and selenium. Among these, arsenic (As) is one of the most prominent pollutant and is a persistent Group 1 human carcinogen (Kaur et al. 2011). The crisis involving the presence of arsenic in water and soil is rampant in parts of China, USA, Europe and Southeast Asia including parts of India. Arsenic contamination in India has been reported from Chhattisgarh, Bihar, Assam, West Bengal and Uttar Pradesh (Shrivastava et al. 2015; Satyapal et al. 2018). Natural processes as well as anthropogenic activities are accountable for the release of arsenic into the environment. Primarily human activities such as usage of insecticides and fertilizers, fossil fuel combustion, mining etc contributed to elevated levels of arsenic in the environment (Srivastava and Sharma 2013).

The maximum arsenic concentration limit recommended by World Health Organization (WHO) in drinking water and groundwater are 0.01 mg/L and 10 parts per billion (ppb) or 10 $\mu\text{g/L}$ respectively (Graham 1999). But the concentration of arsenic in water and soil is way higher than the permissible limit. The arsenic presence in groundwater of Bengal Delta Plain (West Bengal, India and Bangladesh) ranges from 50 to 3200 $\mu\text{g/L}$ (Bachate et al. 2009). In the Upper/Trans-Ganges Plains covering Punjab in northwestern India, arsenic concentration of surface soils has reportedly varied from 1.09 to 2.48 mg As kg^{-1} . While the mean arsenic contents in soil of central India have been found to be higher than soil of West Bengal and Bangladesh. The lowest level of arsenic in the soil of this region is 3.7 mg/kg (Patel et al. 2005).

The arsenic accumulated in soil have the tendency to leach into water bodies and this could result in detrimental effects on human health through ingestion of contaminated water (Nithya et al. 2011). Arsenic associated malignancies include skin lesions, hypertension, ischemia, some endemic peripheral

vascular disorders, severe arteriosclerosis, neuropathies and noticeably, many types of cancer. The toxicity of arsenite lies in its ability to bind to sulfhydryl groups of cysteine residues in proteins and to deactivate them (Paul et al. 2015).

Studies involving the toxic metal-microbe interactions has gained wide attention. These interactions could result in the biogeochemical cycling of metals and in detoxification of metal-contaminated sites (Abbas et al. 2014). Further the correlation between tolerance to heavy metals and antibiotic resistance is another major cause of concern (Wright et al. 2006; Thomas et al. 2020).

Though in the environment, arsenic is present in many organic and inorganic forms, the most prevailing forms found in soil and aquatic surroundings are inorganic arsenate As(V) and arsenite As(III). The widespread occurrence of arsenic in the ecosystem has led to the evolution of arsenic detoxification system in myriad of organisms (Yamamura and Amachi 2014). The *ars* operon consisting of three to five genes, i.e., *arsRBC* or *arsRDABC* located on plasmids/chromosomes of prokaryotes is well specified and is known to involve in arsenic resistance mechanism (Dunivin et al. 2019). The microbial arsenic detoxification entails the reduction of arsenate to arsenite via a cytoplasmic arsenate reductase (*arsC*). In majority of bacterial groups, the arsenite is extruded by a membrane-associated *arsB* efflux pump encoded by three-gene *arsRBC* operons while others employ *ArsAB* pump encoded by five-gene *arsRDABC* operons (Mukhopadhyay et al. 2002). Reduction of arsenate into toxic and mobile form of arsenite leads to environmental arsenic mobilization via microbial arsenate respiration (Yamamura and Amachi 2014). Documented evidences show that the uptake of arsenic by microbes as unintentional as the arsenate resemble nutrients required by the microbes. At physiological pH, the arsenate is taken up by phosphate transport systems because it resembles phosphate (Yang et al. 2012).

Several documented evidences have reported the isolation of several genera such as *Exiguobacterium*, *Aeromonas*, *Planococcus*, *Bacillus*, *Pseudomonas*, *Escherichia*, *Acinetobacter* that illustrated high tolerance capacity for arsenic (0–100mM arsenate) (Upadhyay et al. 2018; Taran et al. 2019).

The situation demands for sustainable and biogenic option for bioremediation of metal contaminated sites. The present study describes the isolation of arsenic resistant bacteria from a heavy metal effluent contaminated soil and its subsequent characterization. The capability of the isolate to withstand high concentration of arsenic was also determined. For getting better insight into the underlying mechanism of arsenic transformation the gene involved was also studied. In this regard, the isolated strain can be envisioned as a promising and sustainable biogenic bioremediation tool for mitigating As toxicity.

Materials And Methods

1.1 Isolation and screening of bacteria

Soil sample was collected from heavy metal contaminated industrial area (pesticide, herbicide industry) in Delhi NCR. Samples were collected from 1–15 cm depth in a sterilized and sealed polyethylene bags and were preserved at 4°C till further use.

The isolation of cultures was done at different concentrations (500 – 10,000 ppm) of sodium arsenate using the modified method of Saxena and Singh 2011. The strain which survived at the highest concentration of sodium arsenate was maintained by subculturing. Pure cultures were used for further studies.

1.1.1 Screening of transforming property of the isolate

The ability of the selected bacterial isolate to oxidize and reduce arsenic was tested using the modified method of Dey et al. 2016.

1.1.2 Physicochemical characterization

The selected pure culture of arsenic resistant isolate was initially characterized by microscopic examination, biochemical and molecular characterization.

1.1.3 Molecular characterization

a) Genomic and Plasmid DNA isolation

The genomic DNA was isolated using the standard phenol-chloroform extraction method while plasmid DNA was isolated according to Anderson and McKay 1983. The isolated DNA was run on 0.8% agarose gel and documented subsequently.

b) PCR amplification of 16S rDNA

Bacterial isolate with high arsenic tolerance capacity was selected for molecular identification by 16S rDNA sequencing. PCR amplification of 16S rDNA gene of bacterial genome was performed using specific forward (5' – AGAGTTTGATCMTGGCTCAG – 3') and reverse (5' – TACGGYTACCTTGTACGACTT – 3') primers. The amplification reaction was carried out using the procedure of Sreedharan et al. 2019.

c) Gene sequencing and strain identification

The gel eluted amplified DNA fragment was sequenced bi-directionally in ABI3500 Genetic Analyzer. Accomplishment of sequencing reaction was done using Big Dye Terminator version 3.1 following the manufacturer's protocol. The procured 16S rDNA sequence was submitted to nucleotide blast (blastn) at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) averse to the nucleotide database to recover homologous sequences to recognize the strain to the generic level.

For phylogenetical analysis, the bacterial 16S rRNA gene sequences from this work and other sequences retrieved from database were aligned using System Software aligner. The phylogenetic analysis was made using the neighbor method using Jukes-Cantor Correction.

d) PCR and sequencing of *arsC* gene

The *arsC* gene amplification was performed using the specific degenerated forward (5' – ATGAGCAACATYACCATTTATCACAAC–3') and reverse (5'– MTTCA YRCGVTTACCTTWWTCATCAAC – 3') primers from genomic DNA of strain RSC3. The amplification reaction was done as previously described by Porwal and Singh 2015. The gel eluted amplified DNA fragment was sequenced bi-directionally in ABI3500 Genetic Analyzer. Accomplishment of sequencing reaction was done using Big Dye Terminator version 3.1 following the manufacturer's protocol. The gene sequence thus obtained was analyzed based on homology analysis using NCBI BLASTn and was submitted to Genbank database.

1.2 Evaluation of minimum inhibitory concentration (MIC)

To determine the level of arsenic resistance, MIC of the selected isolate was determined. The MIC value of the selected isolate for sodium arsenate was assessed according to the clinical and laboratory standards institute (CLSI) protocol. Bacterial inoculum was prepared using Mueller-Hinton broth. The turbidity of the bacterial inoculum was adjusted to 0.5 McFarland turbidity standards (approx. 10^8 CFU/mL). Two-fold serial dilutions of the sodium arsenate in the range of 500-6000ppm were prepared and inoculated with standardized inoculum. Control tubes were maintained without arsenic. The MIC was determined after 24h of incubation at 30°C by observing the visible turbidity. Optical density was measured spectrophotometrically at a wavelength of 600nm. All the experiments were carried out in triplicates. The MIC was defined as the lowest concentration of arsenate that suppressed visible growth of bacteria.

1.3 Cell growth rate kinetics

To study the effect of arsenic on bacterial growth and dry weight, inoculum (1 mL) from an exponentially growing culture (mother culture) was added to 100 mL Nutrient broth supplemented with 500, 1000, 1500 ppm of sodium arsenate. The nutrient broth without arsenic acted as the control. The culture was incubated at 37°C at 120rpm up to 96h. At every 4h time interval samples were withdrawn from each conical flask and the growth rate was measured as absorbance at 600 nm using UV-Vis Spectrophotometer. For plate count results, after every 12h, 100µL of the culture from each broth was serially diluted up to 10^{-8} and was spread on the nutrient agar plates for CFU counting. The growth of the bacterial isolate was monitored by measuring the CFU.

The growth absorbance of *Enterobacter cloacae* (RSC3) was converted into dry biomass using linear coefficient derived from growth absorbance vs. dry biomass (X), the cell growth was follows first-order kinetics.

$$\frac{dX}{dt} = \mu X \quad (1)$$

$$dX/X = \mu dt \quad (2)$$

when integrating Eq. (2) with limit t_0 (initial time) to t (a time when maximum biomass reached), it becomes

$$\ln X - \ln X_0 = \mu(t_0 - t) \quad (3)$$

Where X_0 – initial biomass, X – biomass at time t , and Eq. (3) can be rewritten as

$$\mu = \ln (X_0/X) / \Delta t \quad (4)$$

The specific growth rate (μ) was calculated from the slope of a semi- logarithmic plot of dry biomass $\ln(X/X_0)$ vs. time (Duraishamy et al. 2020).

1.4 Determination of antibiotic sensitivity

Antibiotic sensitivity test was performed using Kirby-Bauer disk diffusion susceptibility test (Benkova, et al., 2020). Different standard antibiotic disks (2–30 mcg), chloramphenicol, kanamycin, vancomycin, tetracycline, nalidixic acid, neomycin, amoxyclave, doxycycline, fusidic acid, clindamycin, ampicillin, azithromycin, erythromycin, sparfloxacin, norfloxacin, streptomycin, rifampicin, gentamycin, trimethoprim, gatifloxacin, ofloxacin, ciprofloxacin were placed on the nutrient agar plates spread with the bacteria and were allowed to incubate at $37^\circ\text{C} \pm 1$ for 24h. Results were analyzed on the basis of size of halo zone around the antibiotic disks.

1.5 Analysis of resistance to other heavy metals

The arsenic resistant bacterial isolate was analyzed for resistance to various other heavy metals which includes Lead, Nickel, Mercury, Cadmium, Selenium, Zinc, Tin, Antimony, Arsenate and arsenite of 1000ppm concentration standard solution. Overnight culture from nutrient agar was inoculated in nutrient broth containing different concentrations of heavy metals (10 to 60ppm), separately. The minimum inhibitory concentration (MIC) of the culture was determined after 24h of incubation at 37°C by observing the visible turbidity. The MIC was defined as the lowest concentration of heavy metals that suppressed the visible growth of bacteria.

1.6 Deciphering the mechanism of arsenic efflux:

To study about the mechanism of arsenic efflux, bacteria was inoculated (approx. 10^8 CFU/mL) in nutrient broth with 1500 ppm of sodium arsenate. The nutrient broth without arsenic acted as the control. The culture was incubated at 37°C , 120rpm for 24h. The sample were withdrawn after 8h and 16h for further assay.

1.6.1 Scanning electron microcopy (SEM) and Energy dispersive x-ray spectroscopy (EDX) analysis

In order to examine the cell morphology of arsenic treated as well as untreated cells SEM was done at 15.00 kV EHT and magnification of 6160X [Zeiss EVO40] using the procedure of Mitra et al., 2021. The

sample was further analyzed for determining the elemental and chemical percentage of arsenic by energy dispersive Xray spectroscopy (EDX) [PANalytical Epsilon 5].

1.6.2 Transmission Electron Microscopy Analysis (TEM)

In order to confirm the intracellular arsenic accumulation, the arsenic treated as well as untreated cells were analyzed using high resolution transmission electron microscope (HRTEM) (Philips, CM-10 model).

1.6.3 Atomic absorption spectroscopy (AAS) analysis

AAS was performed in order to estimate the concentration of arsenic in the selected isolate. The actively grown cells were inoculated in the medium containing 500 ppm arsenic and also in medium without arsenic serving as control. The flasks were then kept for shaking at 37°C at 120rpm. The cells were drawn at different time interval of 0, 4, 8, 12, 16, 20 and 24h for centrifugation at 8000rpm for 20 min at 4°C. After centrifugation, supernatant and pellet were separated, and the pellet was allowed to sonicate. The supernatant and pellet samples were then analyzed for the presence of arsenic by flame atomic absorption spectrophotometer.

Results

2.1 Characterization and identification of the strain

From the metal contaminated site, 118 different bacterial strains were isolated from heavy metal contaminated site. A total of 25 different arsenic tolerant bacterial strains were isolated using enrichment experiment. Out of the 25 isolates, only 3 isolates were able to survive in nutrient agar amended with 6000ppm of sodium arsenate. Among the 3 isolates, one isolate RSC3 that showed the maximum growth at high concentration of arsenate was chosen for the further studies.

The selected isolate RSC3 was screened for its arsenic transforming property and the results showed that it possessed the ability to reduce As(V) as observed by the silver nitrate test (Fig. 1). The arsenic transforming ability of the isolate resulted in the transformation of media color to yellow due to the reduction of arsenate to arsenite. However, the isolate lacks the ability to oxidize arsenite to arsenate. The morphological and microscopic studies revealed it to be Gram negative non-sporulating bacilli. The biochemical results indicated that it belongs to *Enterobacteriaceae* family (Supplementary Table 1).

Taxonomic identification of the isolated strain (RSC3) was determined by analyzing the 16S rDNA gene sequence data with NCBI BLAST database. Blast search using the 16S rDNA gene sequence revealed its affiliation to *Enterobacter cloacae* (Fig. 2). The 16S rDNA nucleotide sequence of the isolate was submitted to NCBI database under the GeneBank accession number (MN904978.1). The strain RSC3 exhibited 99.44% similarity with *Enterobacter hormaechei strain Y2152 plasmid pHI2-2152*, complete sequence. The taxonomic assignment was in accordance with the phenotypic and biochemical analysis.

PCR and sequencing of *arsC* gene

The property of reduction of arsenate to arsenite by the isolated strain revealed presence of the gene in the strain. The gene responsible for reduction was screened in the isolated genomic DNA as no plasmid was isolated from the selected strain. Primer for the *ars* gene retrieved amplicon of 364bp from the isolated genomic DNA (Fig. 3).

The obtained sequence was compared with the available database using BLAST n search, which revealed that the amplicon contains a partial *arsC* gene sequence.

2.2 Evaluation of minimum inhibitory concentration (MIC)

The resistance to arsenate was tested to determine the potential of the isolated bacteria for bioremediation of arsenic. Microbial resistance to arsenate was determined by visible growth after 24 hours in Mueller-Hinton broth amended with varying concentrations of sodium arsenate.

From the recorded optical density, it was observed that the growth of the isolate decreased with increasing arsenate concentration up to 5000 ppm and ultimately stopped growing at 6000 ppm of arsenate. The minimum inhibitory concentration (MIC) of the isolate was 6000 ppm for arsenate.

2.3 Evaluation of Growth kinetics

Time course assay of *Enterobacter cloacae* (RSC3) revealed that the growth in presence of arsenic at concentration range from 500–1500 ppm was found to be 50 mg L⁻¹ of biomass around 24 h (Fig. 4). The biomass at different arsenic concentration measured every 4h at OD 600 nm was converted into dry biomass (mg L⁻¹) to calculate growth rate (Fig. 5). The specific growth rate (μ) was calculated as 0.55 h⁻¹.

The growth response of RSC3 in the presence of As(V) ions was defined in terms of colony forming unit (CFU) on nutrient agar plates. The presence of the arsenic ions resulted in the lengthening of the log phase as evident from Fig. 6.

The growth of the isolate RSC3 decreased with the increase in concentration of arsenate. The shift in the concentration of arsenate from 500ppm to 1000 ppm resulted in 1.5 log reduction during maximum bacterial growth. Irrespective of arsenic concentration, the strain exhibited maximum growth around 50–55h of incubation but after that its growth decreased and it entered the decline phase.

2.4 Determination of antibiotic sensitivity

The Sensitivity and/or resistance to a particular antibiotic was determined by growth tests on solid medium plates containing disc of antibiotic. The results revealed that the isolate RSC3 was resistant to all the protein synthesis inhibitor antibiotics used in the experiment except Gentamycin Table 1. The isolate also demonstrated intermediate and complete resistance against the various cell wall and DNA synthesis inhibitors.

Table 1
Antibiotic resistance of *Enterobacter cloacae* RSC3

S.no.	Biochemical test	Result
1.	Indole	-ve
2.	Methyl red	-ve
3.	Voges– Proskauer’s	-ve
4.	Citrate utilization	+ve
5.	Lysine utilization	-ve
6.	Ornithine utilization	+ve
7.	Urease	-ve
8.	Phenylalanine deamination	-ve
9.	Nitrate reduction	+ve
10.	H ₂ S production	-ve
11.	Glucose	+ve
12.	Adonitol	-ve
13.	Arabinose	+ve
14.	Lactose	+ve
15.	Sorbitol	+ve
16.	Mannitol	+ve
17.	Rhamnose	-ve
18.	Sucrose	+ve
S.no.	Antibiotics	Result
1.	Erythromycin	+
2.	Streptomycin	+
3.	Chloramphenicol	+
4.	Tetracycline	+
5.	Rifampicin	++
6.	Ofloxacin	++

+ = resistant; ++ = intermediate resistance; +++ = sensitive

S.no.	Biochemical test	Result
7.	Sparfloxacin	++
8.	Gatifloxacin	++
9.	Ciprofloxacin	++
10.	Ampicillin	+
11.	Kanamycin	+
12.	Doxycycline hydrochloride	+
13.	Azithromycin	+
14.	Vancomycin	+
15.	Nalidixic acid	+
16.	Neomycin	++
17.	Gentamycin	+++
18.	Amoxyclav	++
19.	Clindamycin	+
20.	Norfloxacin	+
21.	Fusidic acid	+
22.	Trimethoprim	+
+ = resistant; ++ = intermediate resistance; +++ = sensitive		

2.5 Analysis of resistance to other heavy metals

In addition to arsenic resistance, RSC3 grew very well in the presence of a variety of heavy metals. Multi-resistance to zinc, cadmium, selenium and nickel was observed and exhibited MIC in the range of 30–50 ppm. The isolate was showing less tolerance for lead, tin, antimony and mercury of upto the concentration of 10 ppm. MICs for different metals are shown in Fig. 7.

2.6 Deciphering the mechanism of arsenic efflux:

2.6.1 Scanning electron microscopy (SEM) analysis and Energy dispersive x-ray spectroscopy (EDX) analysis

The Scanning electron microscopic (SEM) studies were performed on the bacterial isolate grown in the presence and absence of arsenic. SEM images are shown in Fig. 8. The images illustrated minor changes in cell morphology and size in terms of reduction in cell size with aggregation of cells when the isolate was grown in the presence of arsenic.

When the cells were exposed to arsenic some morphological features were evident from SEM micrograph. The cells were observed as a uniform mixture of spherical and elongated cells in packed aggregation as well as individuals. The arsenic which entered the cell was effluxed out of the cell due to the presence of active efflux mechanism of resistance in the isolate as confirmed by AAS analysis.

EDX analysis was used to characterize their elemental composition. The presence of arsenic was also confirmed by EDX analysis. The EDX analysis exhibited an EDS signal corresponding to arsenic peak which was perceived in the presence of arsenate treated cell (Fig. 9), however no such peak was observed in case of control.

2.6.2 Transmission Electron Microscopy Analysis (TEM)

In order to confirm the intracellular arsenic accumulation, the arsenic treated as well as untreated cells were observed by transmission electron microscope (TEM). The presence of electron dense layer in the center of the cell after 8h of incubation (Fig. 10a) and its migration towards the walls of the bacterial cell after 16h of incubation (Fig. 10b) was noticed in cells treated with arsenic. Moreover, we also detected insoluble precipitates of arsenic in both TEM analysis (Fig. 10a and 10b).

2.6.3 Atomic absorption spectroscopy (AAS) analysis

The AAS results revealed higher concentration of arsenic in the pellet of selected isolate at the start of log phase which then decreased towards the end of log phase while trend reversal was detected for the concentration of arsenic in the supernatant, i.e. there was a lower concentration of metal in the initial phase and then it improved with time illustrating the efflux of metal out of the cell (Fig. 11). This was in concurrence with the SEM results which indicated that there was minor change in the cell morphology of the isolate, indicating towards no accumulation of the metal and hence supporting the efflux mechanism of the isolate RSC3.

Discussion

Microbe-mediated transformation of arsenic has tremendous potential in bioremediation of contaminated soil and aquifers. In microbial communities, the implication of arsenic presence has catalyzed the development of survival instinct in the form of detoxification mechanisms. In this study, the multi-dimensional abilities of indigenous bacteria (RSC3) isolated from arsenic contaminated industrial sites has been deciphered.

The arsenate reducing RSC3 was characterized to be *Enterobacter cloacae* using 16S rDNA gene sequence. Prior studies by Anderson and Cook (2004) have resulted in isolation of seventeen bacterial strains including *Bacillus licheniformis*, *Bacillus polymyxa*, etc. which were able to resist up to 100 ppm arsenic. In a study by Paul et al. 2015, 60% of the isolated strains demonstrated arsenate reductase activity. Abbas et al. 2014 have isolated a strain (MNZ1) which showed homology with *Enterobacter* sp. Dey et al. 2016 have reported arsenic oxidizing bacteria namely *Bacillus* sp. and *Aneurinibacillus aneurinilyticus*.

The detection of partial *arsC* gene sequence in the bacterial isolate has demonstrated its potential to reduce arsenate to arsenite. Bachate et al. 2009 have also amplified A 275 bp fragment of putative *arsC* gene from *Bacillus* sp. Rice C. The presence of *arsBC* gene pair is reported in the chromosomes of gram negative bacteria. In case of bacteria such as *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* *arsC* genes was not found to be associated with *arsB* genes. While on the other hand, *P. aeruginosa* reportedly had a second *arsC* gene apart from the one existing within the *arsRBC* operon (Mukhopadhyay et al. 2002).

The property of selected isolate RSC3 to reduce As(V) was in concurrence with the already reported paper which has also described *Enterobacter cloacae* as an arsenate reducing bacteria (Selvi, et al. 2014). In another work, Banerjee et al. 2011 have reportedly isolated 10 different bacterial strains out of which two *Pseudomonas* sp. (RJB-1, RJB-3) and one *Vogesella* sp.(RJB-C) showed the ability to reduce As(V).

The possibility of the isolate harboring antibiotic resistance was analyzed and results obtained from this study indicating the resistance of *Enterobacter cloacae* towards ampicillin and amoxycylav is consistent with previous reports (Selvi et al. 2014). The isolated strain RSC3 was also found to be resistant to chloramphenicol while most isolates of the *E. cloacae* complex are susceptible to fluoroquinolones, trimethoprim/sulfamethoxazole, chloramphenicol, aminoglycosides, tetracyclines, piperacillin–tazobactam and carbapenems (Mezzatesta et al. 2012). This indicated that may be *Enterobacter cloacae* RSC3 have also acquired resistance for chloramphenicol. These results could be interpreted as that resistance can be conferred by a plasmid or chromosome-encoded resistance and/or by a system not yet described. In this study as no plasmid was isolated, it indicated the possibility of having coexistence of both types of determinants for antibiotic and heavy metals in the same genetic element (chromosome) which may allow antibiotic resistance to be selected upon heavy metal selective pressure in the contaminated environment (Farias et al. 2015).

The MIC value for arsenate was 6000 ppm for our isolate which has been comparable to Dey et al. 2016 who isolated *Bacillus* sp. KM02 and *Aneurini bacillus aneurinilyticus* that could tolerate 4500ppm of arsenate. Moreover, an isolate of *Providencia rettgeri* has also been reported that can tolerate As (V) upto 10,000µg/mL (Kale et al. 2015). Higher tolerance towards arsenic apt its possible use in reclaiming the different contaminated sites.

The growth pattern of *Enterobacter cloacae* RSC3 in the presence of arsenic demonstrates that it could reproduce and survive despite the metal stress. In this study, we are reporting the strain is able to grow comfortably at 1500 ppm upto 60h indicating this is one of the effective arsenic tolerating strain reported so far. The increase in the duration of lag phase may be due to the toxic effect of arsenic on the functionality of bacteria leading to the arduous task of repairing and rectification of the processes affected by the metalloid presence. Simultaneously, the duration of log phase observed in this study was also quite lengthy. It could be due to the interference in phosphate transport system for the uptake of arsenic leading to the extension of logarithmic growth. Paul et al., also reported a growth response of

KUMAs15 at different concentrations of arsenate and arsenite with lengthened lag phase but the maximum growth obtained for KUMAs15 was after 28–30h of incubation (Paul et al. 2018).

In addition to arsenic resistance, multi-resistance to zinc, cadmium, selenium and nickel was observed for *Enterobacter cloacae* RSC3. These results remain in agreement with those reported previously by Selvi et al. 2014. Several other reports concerning the evaluation of multi-resistance in arsenic resistant bacteria is also available (Banerjee et al. 2011; Biswas et al. 2019; Rahman et al. 2014). The genes responsible for imparting heavy metal resistance are rampant in micro-organisms and this phenomenon of polymetal resistance can be attributed as an adaptation of microbes to the presence of varied metal and metalloid ions in its habitat. Pal et al. 2015 identified general patterns for which biocide/metal resistance genes (BMRGs) and antibiotic resistance genes (ARGs) that tend to occur together. They revealed that genome with BMRGs carried along ARGs and arsenic, cadmium, nickel, mercury, copper, silver, iron, zinc and cobalt are probable co-partners in selecting microbes resistant to aminoglycosides, sulfonamides, beta-lactams and tetracyclines. The heavy metal tolerance properties of the isolate make it to be a good candidate for various biotechnological and bioremediation processes.

The arsenate transportation and efflux of arsenic by the *Enterobacter cloacae* RSC3 was verified by TEM, SEM, EDX and AAS analysis. The morphological characteristics of arsenic exposed cells under SEM depicted minor changes in cell morphology and size. There was reduction in cell size with aggregation of cells as a mode of bacterial response towards arsenic stress. These changes in cell morphology can be interpreted as relative decrease in the surface area/volume ratio to reduce the effects of arsenic toxicity by decreasing the attachment /uptake sites for heavy metals (Chakravarty and Banerjee 2008). In accordance with our result, a similar result was observed in a previously reported investigation in which the isolate SW2 also reduced in size in comparison to that of control cells (Dey et al. 2016). In contrast, the studies of Banerjee et al. 2011 reported the formation of long chains of bacteria in presence of arsenic while that of Saluja et al. 2011 described 2 strains (AG24 and AGM13) that showed no eminent changes in the cell morphology and size, when the strains were grown in presence of arsenic.

Structural modifications of arsenic exposed *Enterobacter cloacae* RSC3 observed by TEM examination were in accordance with the reports by Pandey and Bhatt 2015 who reported that increased arsenic accumulation in cells. Further they reportedly found a fourfold increase in bacterial cell volume when grown in the presence of arsenic. The EDX analysis exhibited an EDS signal corresponding to arsenic peak which was perceived in the presence of arsenate treated cell which is contributing to the hypothesis of entry and exit of arsenic in a modified form from the cell. The result of AAS analysis revealed that arsenic which entered the cell was emitted out of the cell due to the presence of active efflux mechanism of resistance in the isolate. Similar results were reported in a study Saluja et al. 2011, in which the 2 strains (AG24 and AGM13) exhibited a similar pattern of efflux of metal from the cell. Altogether these findings emphasized the presence of arsenic efflux system in the isolated strain. Figure 12 depicts the schematic representation of arsenic uptake and efflux of our *Enterobacter cloacae* strain. The arsenic enters inside the bacterial cell along with the phosphate using phosphate transferase system (*pst* ABC). The *arsC* gene responsible for production of arsenate reductase reduces As (V) to As(III). This As (III),

through a series of reaction, is effluxed out through the cell using other *ars* operons. Our hypothesis is ably supported by other scientific evidences which has demonstrated the removal of arsenic by bacterial strains (Yamamura and Amachi 2014; Dunivin et al. 2019; Mukhopadhyay et al. 2002).

Conclusions

Cost effective and biogenic remedy tools to circumvent the environmental arsenic pollution in both lands as well in water is the need of the hour. Indigenous arsenic resistant microbes isolated from the contaminated sources are really cherished in this aspect. The arsenic resistant bacteria isolated in this study was *Enterobacter cloacae* based on phylogenetic analysis of 16S rDNA sequence.

This current work demonstrates the capabilities of the isolated strain to withstand concentrations of upto 6000ppm of arsenic. The strain possessed an active efflux system which was confirmed with SEM-EDX, TEM and AAS analysis. Further multi-resistance to several heavy metals and antibiotics enhances the desirability factor of the isolate for judicial application in developing an *in situ* bioremediation technology.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: Yes

Competing interests: "The authors declare that they have no competing interests"

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Authors' contributions: RB performed the experiments. SMS analyzed the data. RS designed the study and analyzed the data. All authors wrote, read and approved the final manuscript.

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Figures

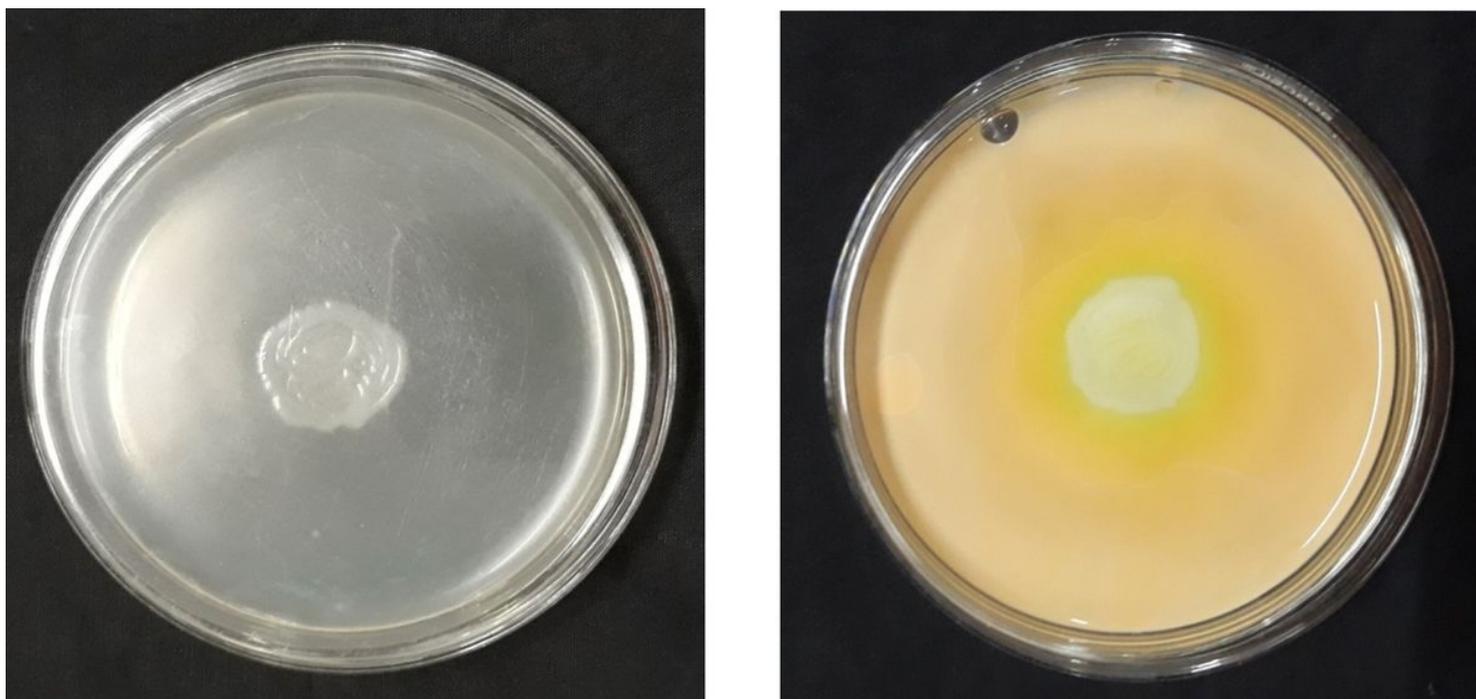


Figure 1

Silver nitrate screening test in sterile nutrient agar plate containing sodium arsenate: (a) without addition of silver nitrate; (b) After addition of silver nitrate solution showing arsenate reducing isolate RSC3

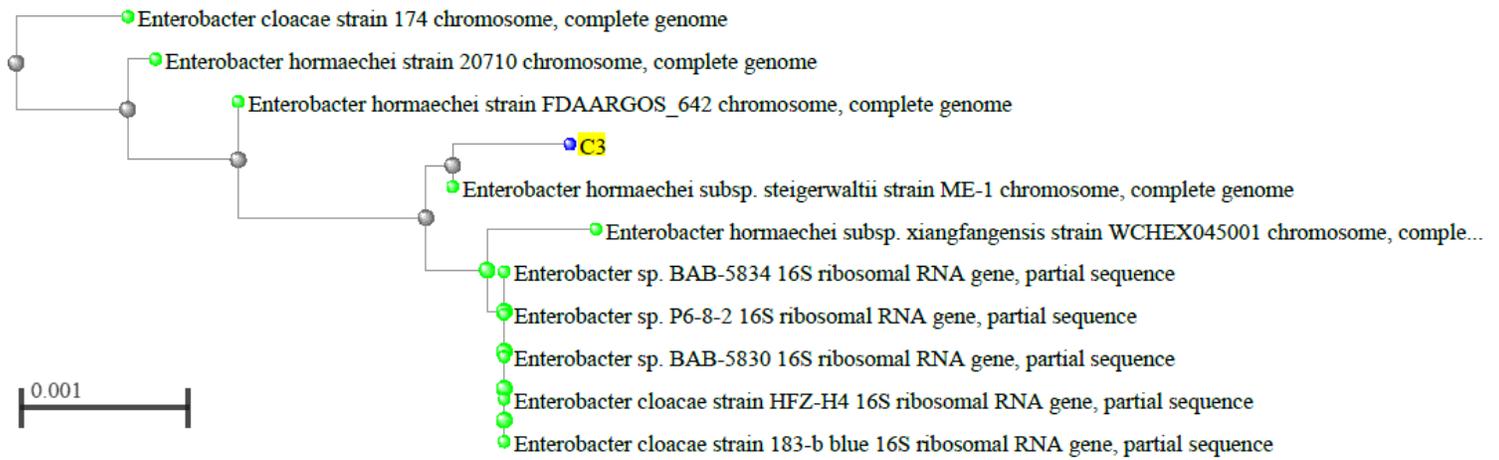


Figure 2

Phylogenetic tree based on 16S rRNA gene sequences depicting relationship between RSC3 and related bacteria.

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TTGAAGCGTTAGGGCTGGCGGAAGACCGCTTTACTGACGAGCAGTTAATCGATTTTATGTTGCAGCAC
CCTGTTCTGATCAATCGCCCTATCGTGGTAACGCCGCTGGGTACCCGGCTATGCCGCCCTTCTGAAGTC
GTGCTGGATATTCTTCCCGATGCGCAGAAAAGCGCGTTCACGAAAGAGGACGGTGAGAAGGTAGTTG
ATGATTAAGGTAACCGCGTGAAGA

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Figure 3

Partial Sequence of arsC gene amplicon of 364bp from the isolated genomic DNA

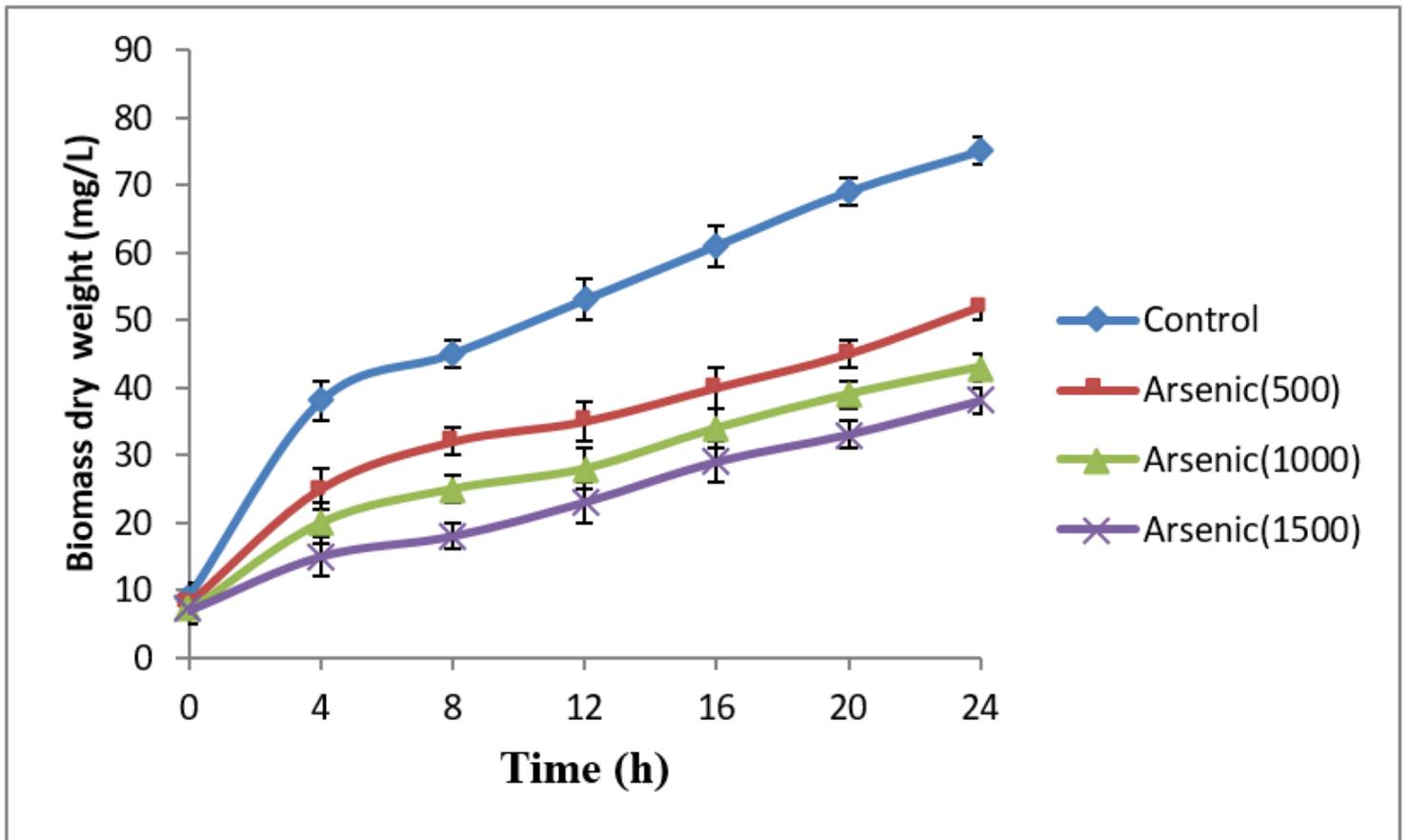


Figure 4

Biomass of RSC3 at a various arsenic concentration.

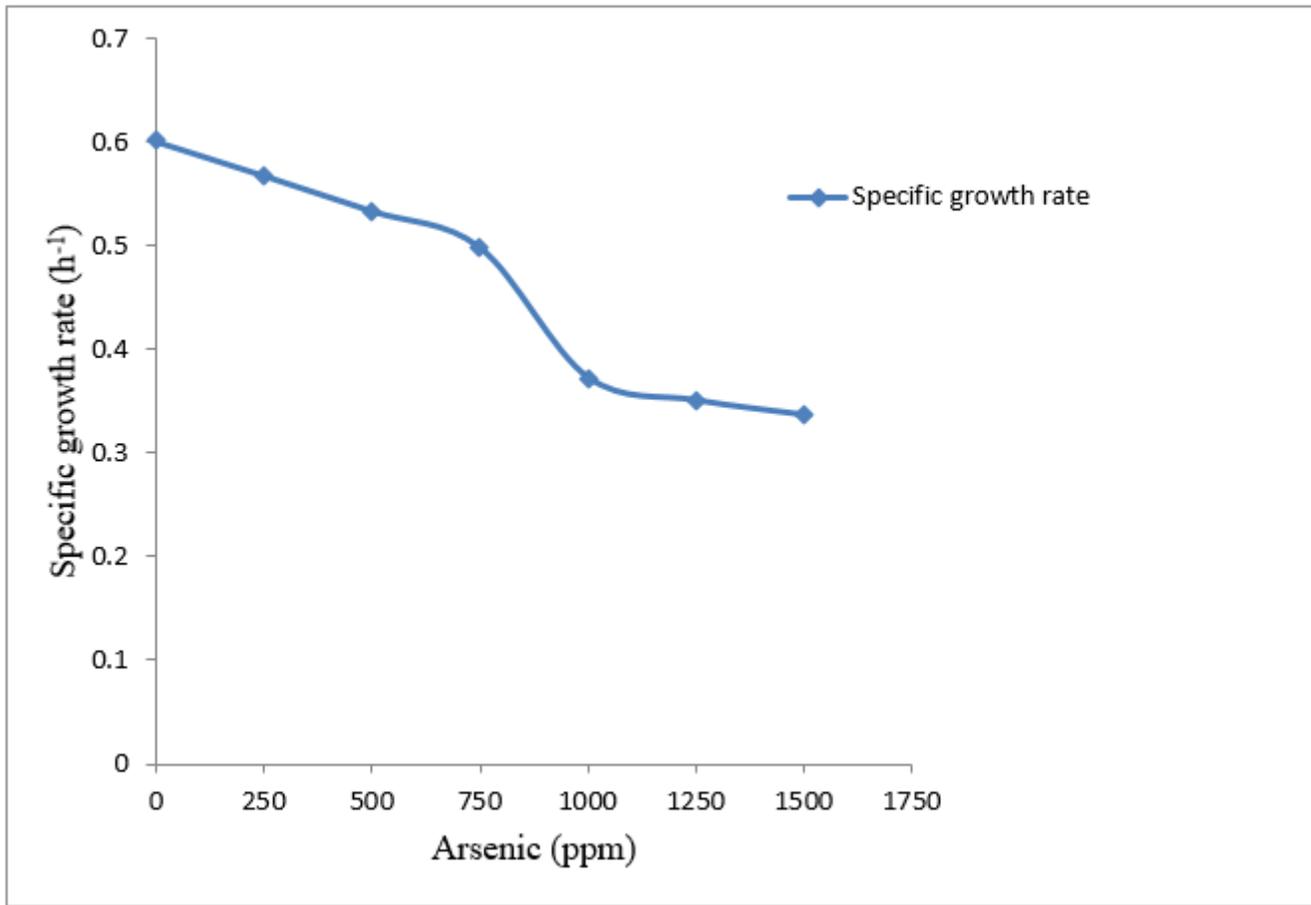


Figure 5

Relationship between specific growth rate and initial arsenic concentration

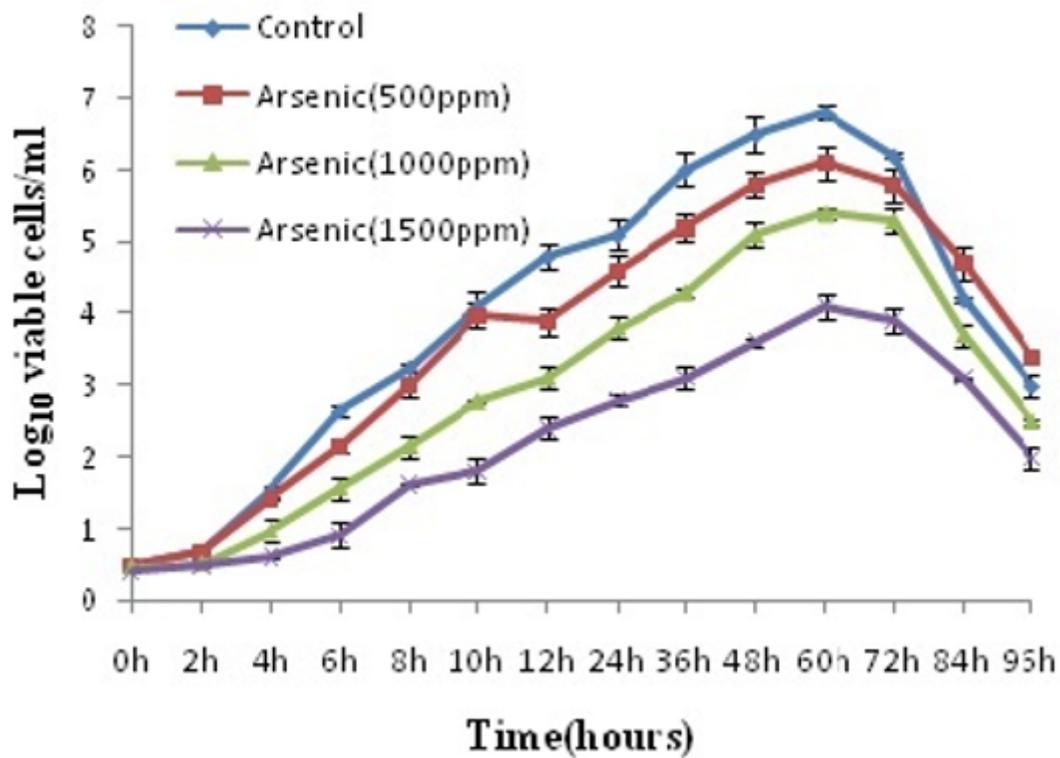


Figure 6

Growth kinetics of RSC3 in media supplemented with 500, 1000 and 1500 ppm of sodium arsenate and without sodium arsenate as control

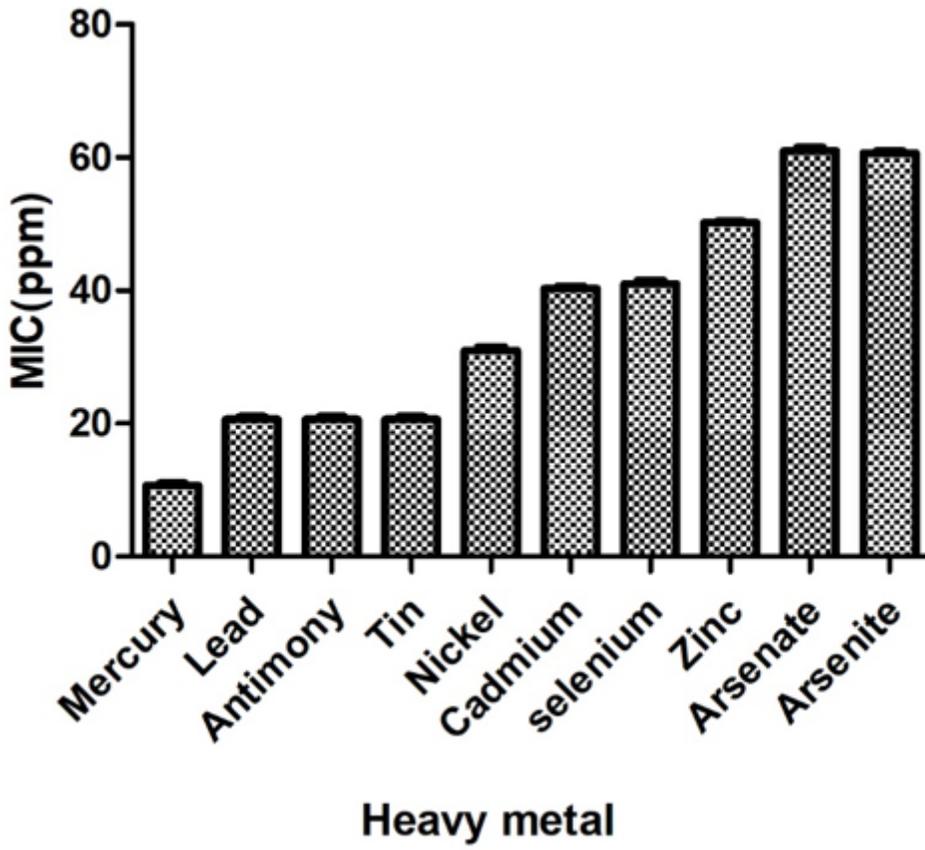
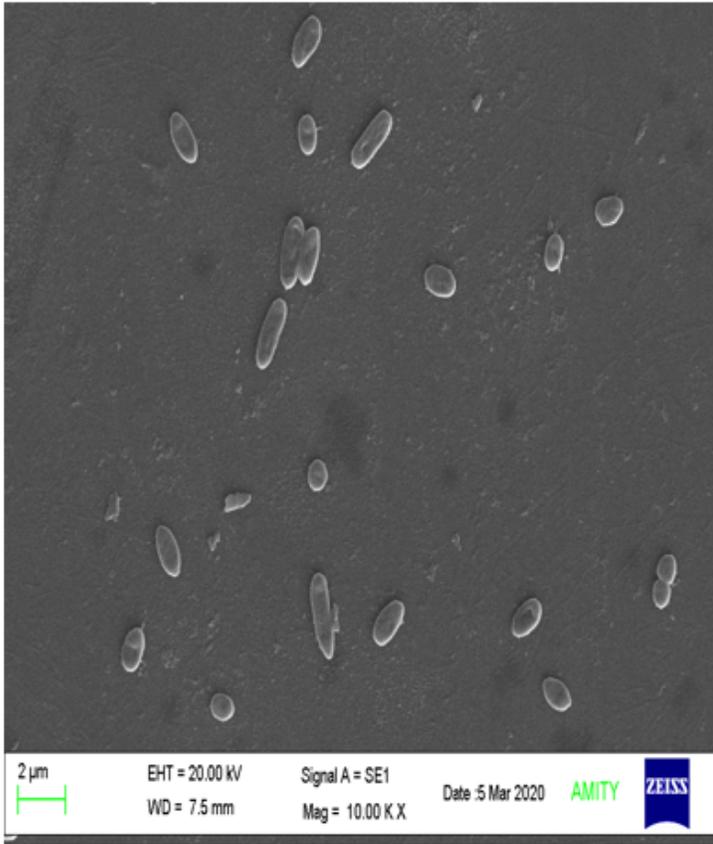
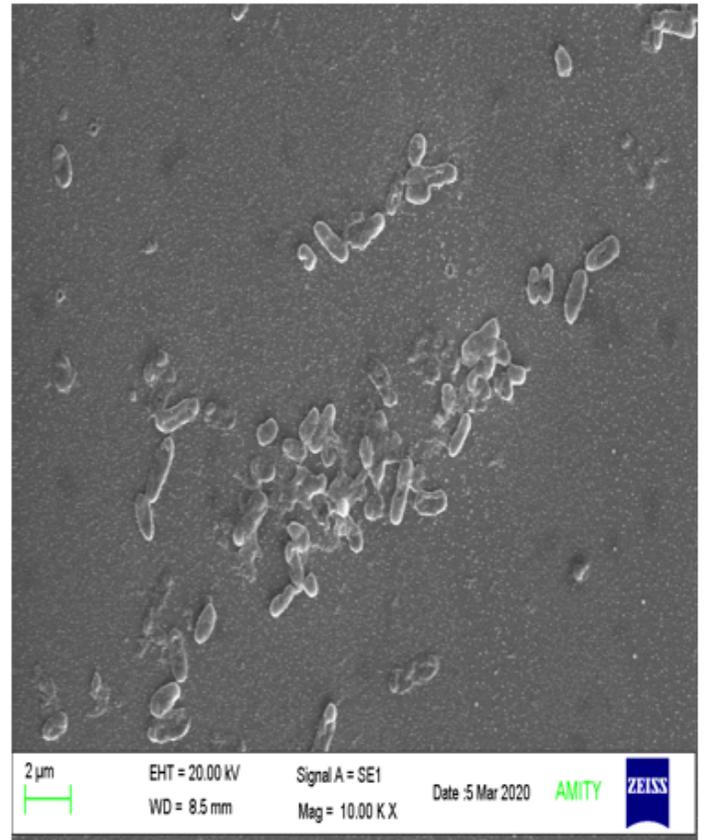


Figure 7

MIC of the *Enterobacter cloacae* RSC3 for different heavy metals (in ppm)



(a)



(b)

Figure 8

(a) Control (without arsenic) (b) In presence of arsenic Scanning electron micrograph showing the effect on the shape and size of Enterobacter cloacae RSC3 in presence and absence of arsenic (a) Control (without arsenic) (b) the effect on the cellular morphology of Enterobacter cloacae RSC3 in presence of arsenic

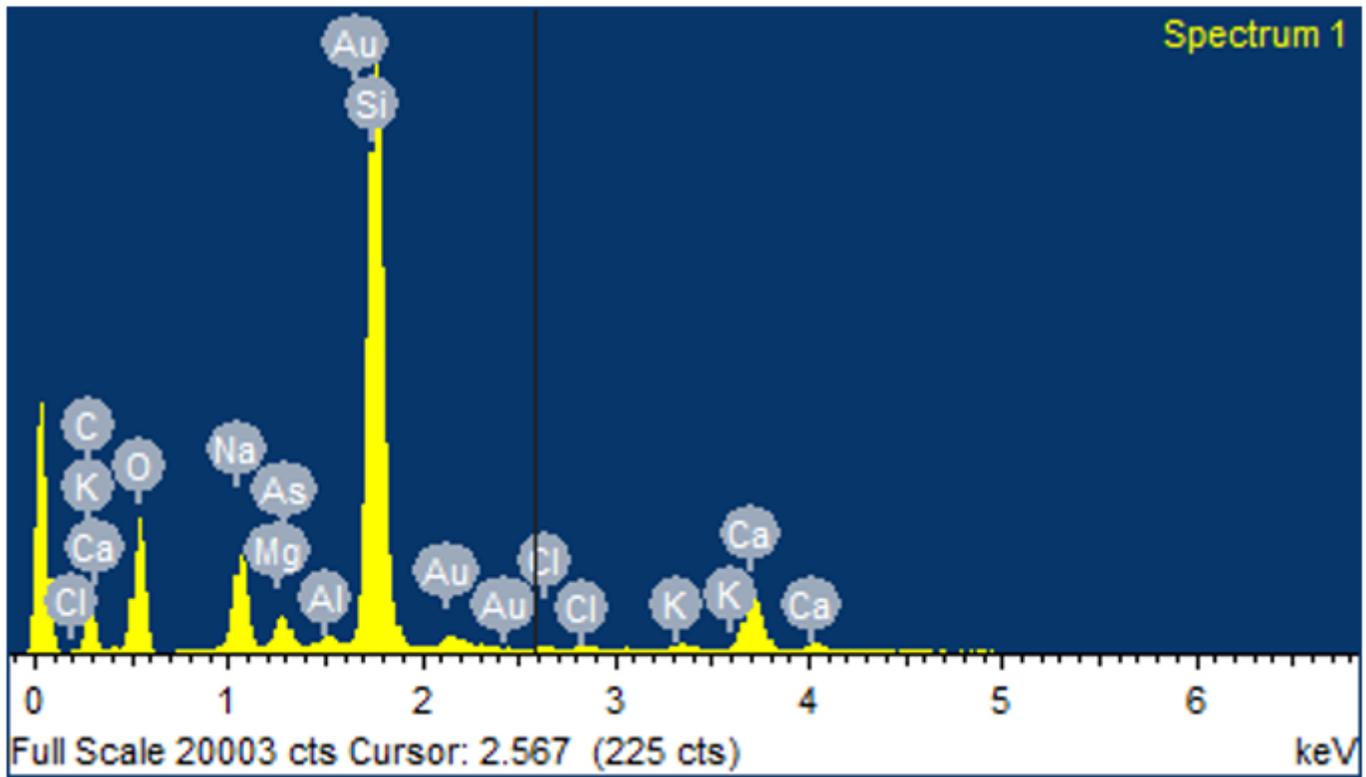
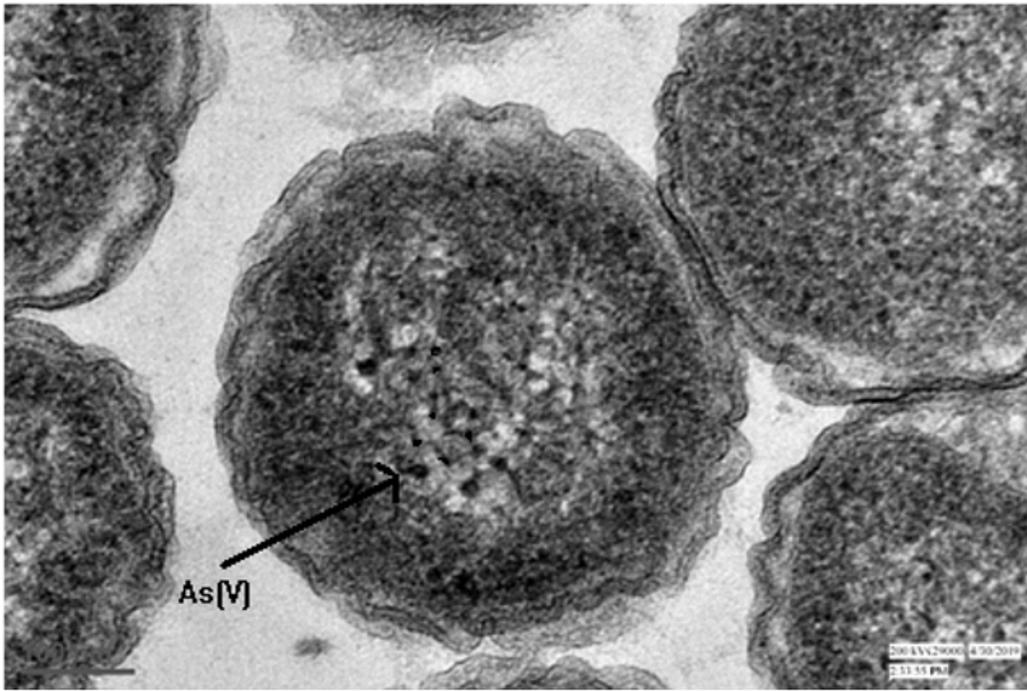
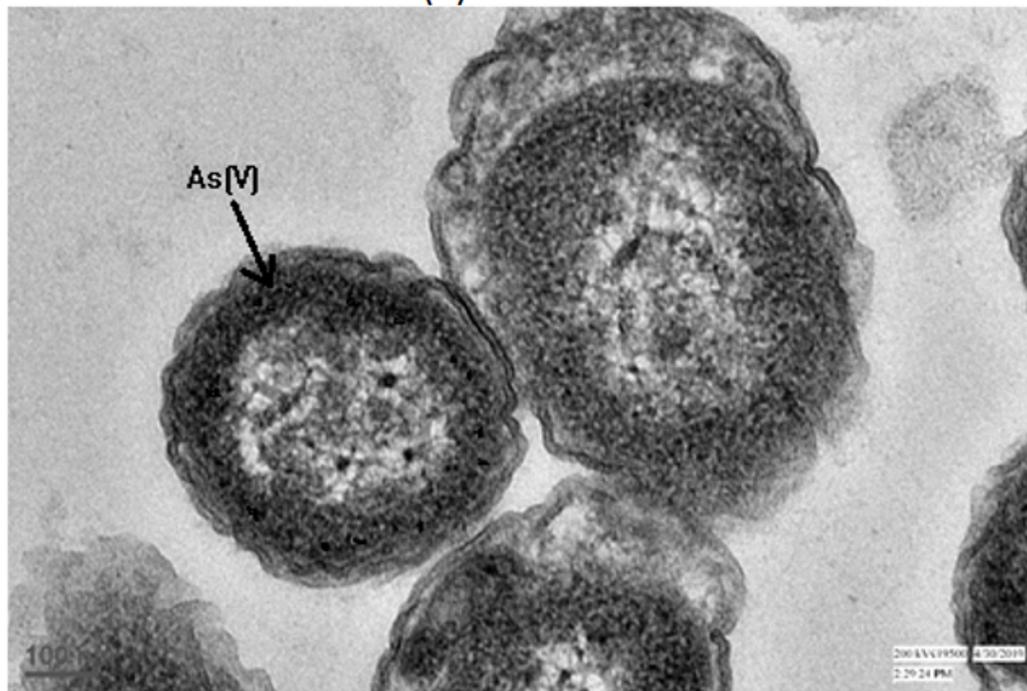


Figure 9

EDX analysis of *Enterobacter cloacae* RSC3 showing presence of arsenic inside the bacterial cell



(a)



(b)

Figure 10

TEM micrograph showing the movement of arsenic inside the bacterial cell a: Presence of arsenic inside the bacterial cell after 8h of incubation b: Movement of arsenic towards the bacterial cell wall after 16h of incubation for efflux

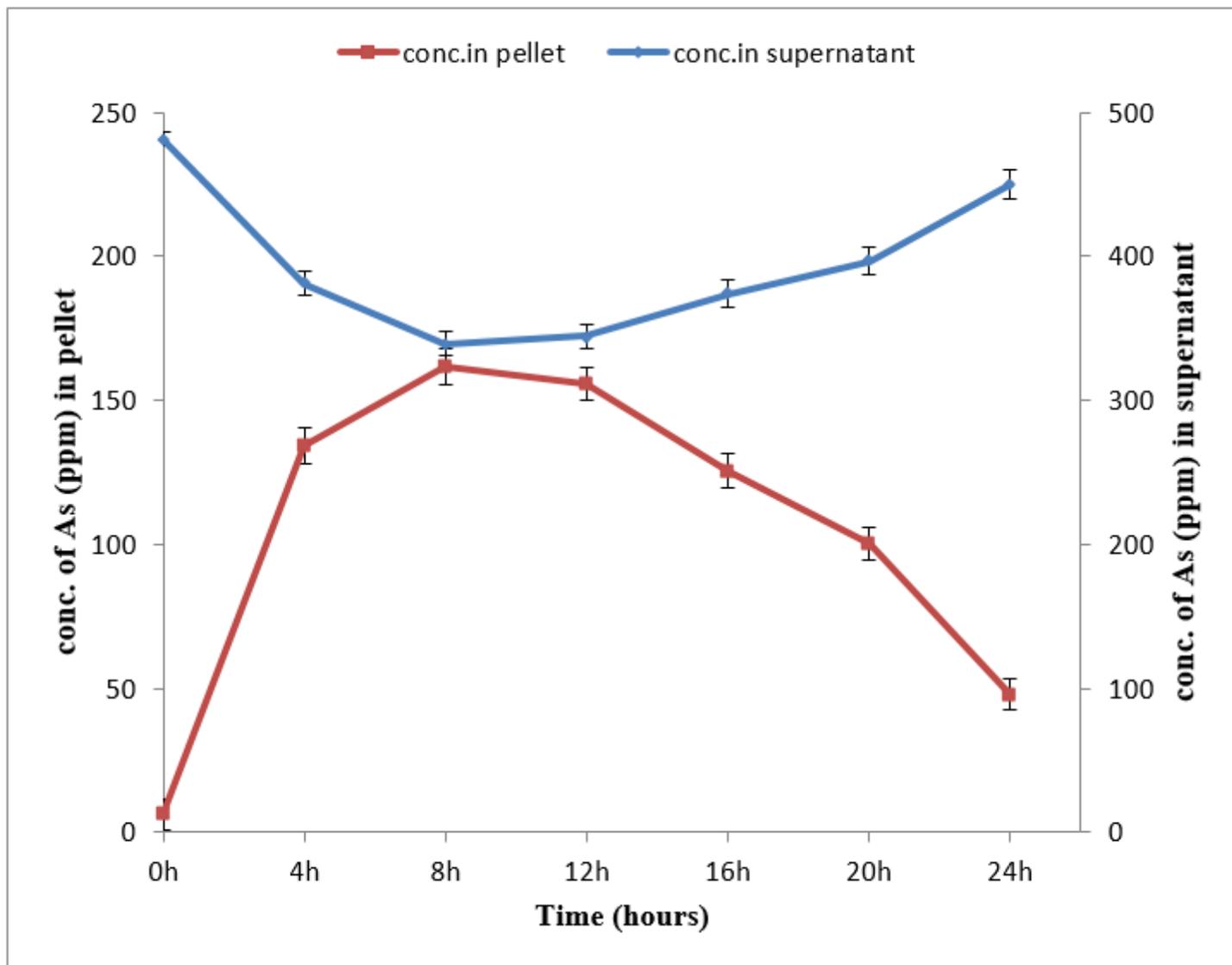


Figure 11

The AAS results showing arsenic concentration in pellet and supernatant fraction of *Enterobacter cloacae* RSC3 at different time intervals illustrating the efflux of metal out of the cell

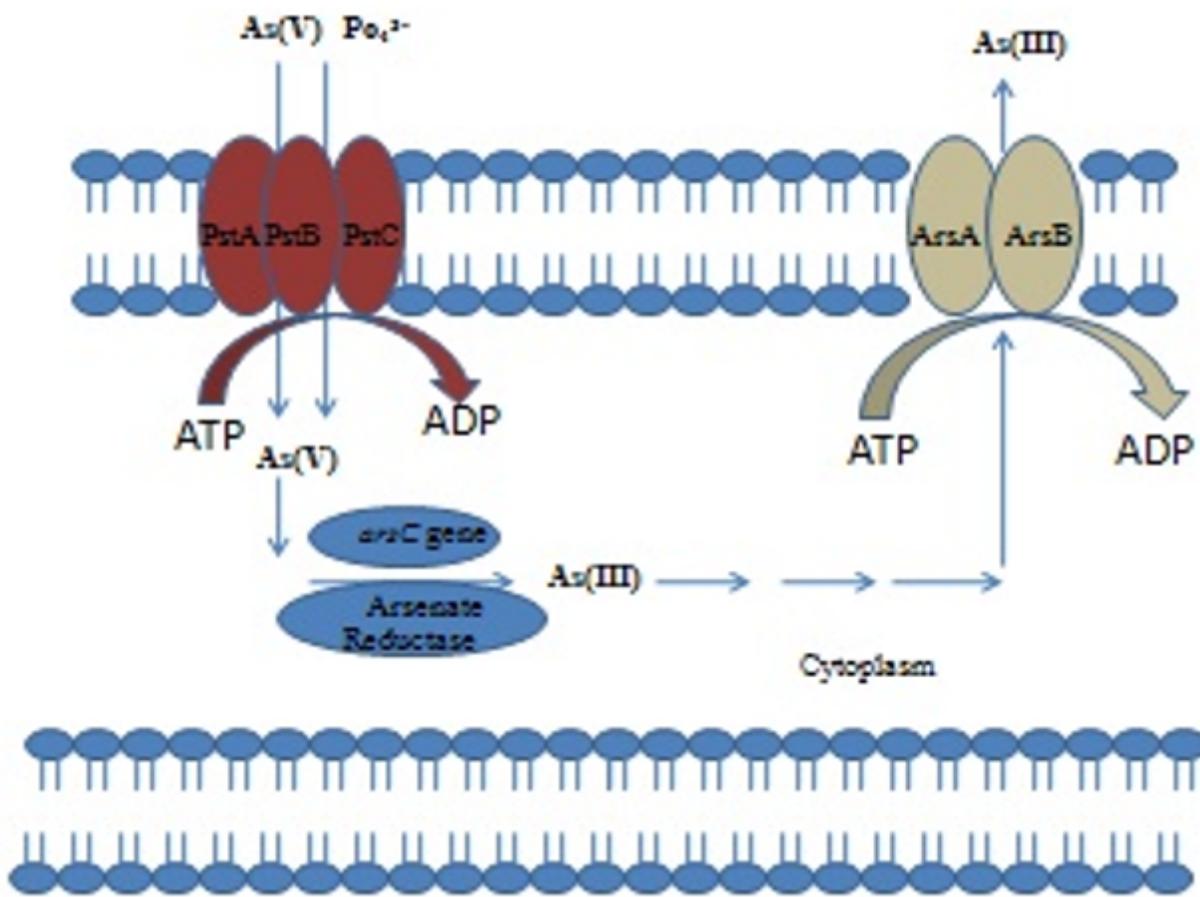


Figure 12

Schematic representation of arsenic uptake and efflux by *Enterobacter cloacae* RSC3.

Supplementary Files

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- [SupplementaryTable1.docx](#)