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Isolation of Arsenic Resistant *Escherichia coli* from Sewage Water and Its Potential in Arsenic Biotransformation

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ABSTRACT

Arsenic contamination in drinking water from ground water poses a threat to the health of a large population in developing countries in Asia. This has sparked great interests in the potential of different microbes in arsenic resistance and removal from water. This study involves isolation of arsenic resistant *Escherichia coli* from sewage water from Kathmandu University and investigation of its attributes. Arsenic resistant *E. coli* was successfully isolated which could survive in high concentration of arsenic. The maximum tolerance of arsenite was 909.79 mg/L (sodium arsenite) and 3120.1 mg/L arsenate (sodium arsenate) which is well above most natural concentration of arsenic in ground water. This particular *E. coli* tolerated multiple heavy metal like silver nitrate, cobalt sulphate, cadmium chloride, nickel chloride, mercury chloride, copper sulphate, and zinc chloride at concentration 20 µM, 1 mM, 0.5mM, 1mM, 0.01 mM, 1 mM, and 1 mM respectively which are concentrations known to be toxic to *E. coli*. Biotransformation of arsenite to arsenate was also checked for by a qualitative silver nitrate technique. This *E. coli* was able to transform arsenate to arsenite. It showed some sensitivity to Ciprofloxacin, Gentamicin and Nalidixic Acid. As *E. coli* and its genome are very widely studied, these particular properties have a lot of potential in microbial remediation or microbial recovery of metals and possible recombination approaches.

Keywords: *Arsenic, arsenic resistance, bacteria, maximum tolerance, heavy metals*

INTRODUCTION

There are more than fifty Arsenic (As) species that have been identified, with most among them mainly observed in aqueous environments [1]. The most important of these in terms of geochemical studies are the inorganic species trivalent arsenite (As III) and pentavalent arsenate (As V), along with methylated As species, monomethylarsenite (MMA (III)), monomethylarsenate (MMA (V)), dimethylarsenite (DMA (III)), and dimethylarsenate (DMA (V)) [1]. The toxicity of arsenic is dependent on its redox state or its binding form. It is known that the organic forms of arsenic are less toxic than the inorganic form [2]. Among the inorganic As species, arsenite is considered to be more toxic [3]. In most ground water the As concentrations are well below the limit set by WHO (10 µg/L) typically below analytical detection limits. Although based on hydro-geological and geochemical parameters As Concentrations can reach up to hundreds of µg/L.

A distressingly large population of developing countries like India, Bangladesh, Nepal, and rural China are at risk of arsenic poisoning due to consumption of arsenic contaminated groundwater due to geogenic sources. Some believe this to be no other than mass poisoning case. As we know arsenic is a poison, a number of taxonomically diverse microorganisms have been known to possess biochemical mechanisms that can either thwart arsenic from entering cells or quickly extrude it back to the environment once it does enter [3]. These biochemical mechanisms for said detoxification are basically centered on redox reactions and changes between the As (III) and As (V) oxidation states which alter the speciation of arsenic found in the surrounding aqueous medium. Many bacteria have been identified that exhibit resistance or can grow in concentrations of arsenic considered lethal [4]. As opposed to artificial chemical approach, microorganisms have been reported to be able to efficiently remove soluble and particulate forms of metals usually from di-

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Table 1. MTC of *E. coli*

Organism	*Arsenite concentration*		*Arsenate concentration*	
	mg/L of $NaAsO_2$	*mM*	*Mg/L of* Na_2HAsO_4	*mM*
E. coli	909.79	7	3120.1	10

Note : - 1 mM Sodium Arsenate solution = 312.01 mg/L = 74.91 mg/L of arsenic

- 1 mM Sodium Arsenite solution = 129.91 mg/L = 74.91 mg/L of arsenic

Figure 1. *E. coli* colonies show green metallic sheen in EMB agar. The green sheen is used to confirm that the bacteria growing is in fact *E. coli*

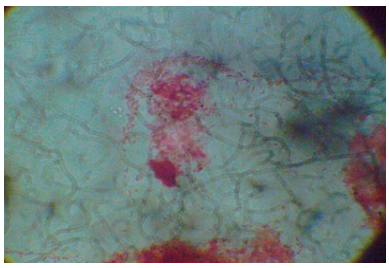


Figure 2. Micrograph of *E. coli* after gram staining

lute solutions through processes of bioaccumulation. Microorganism-based technologies can therefore offer a viable alternative to the conventional techniques of metal removal or recovery which involves chemical or physical approach [5]. Apart from these natural phenomenon that influence arsenic in ground water, microbial activity has also been linked with arsenic mobilization. It has been reported that microbial activity in ground water can control Arsenic contamination by forming biominerals with arsenic [6]. Microorganisms have been known to grow in extreme condition including conditions with excessively high arsenic [7]. This paper evaluates characteristics of arsenic resistant *Escherichia coli* isolated from sewage water from Kathmandu University for its arsenic resistance and removal potential to be utilized in bioremediation.

MATERIALS AND METHODS

Isolation

Escherichia coli from sewage water were isolated by using radiant streaking in EMB media and subsequently culturing to obtain a pure culture. A sample of pure culture of *E. coli* from sewage water were first grown in media containing Arsenite and arsenate at concentrations of 10 µg/L (WHO limit for water). Once the bacteria grew in the media they were gradually subculture in media containing higher concentration of arsenic. Growth in higher concentrations of arsenic indicates selection of arsenic resistant *E. coli*. These bacteria will be grown in nutrient agar and broth supplemented with arsenite and arsenate in the form of sodium salts. *E. coli* shows colonies green metallic sheen in EMB agar which indicates that they are in fact *E. coli* although IMViC test is more thorough.

Determination of maximum tolerance concentration (MTC)

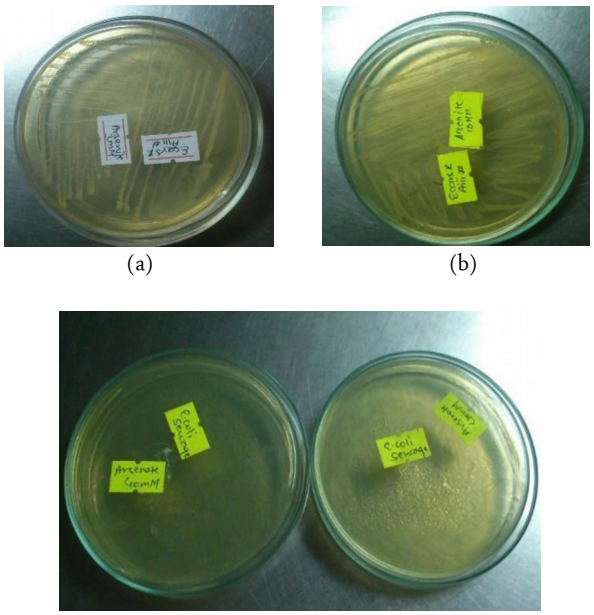
Resistance to arsenate and arsenite was determined for *E coli* by growing them on nutrient agar and nutrient broth with gradually differing concentrations of arsenate and arsenite respectively. Stock solutions of one molarity were prepared for both Sodium arsenite and Sodium arsenate. The media of required molarity was prepared by adding required volume from stock. After inoculation of bacteria in these media they were incubated for at least 48 hours at 30°C. The maximum tolerance concentration was determined by observing visible colonies in agar and turbidity in broth at the highest concentration of arsenic. The tests were carried out in triplicates.

Determination of heavy metal resistance

The bacteria were also studied for resistance to heavy metals other than arsenic. Resistance to other heavy metals was determined for zinc chloride ($ZnCl_2$), copper sulphate ($CuSO_4.5H_2O$), mercuric chloride ($HgCl_2$), nickel chloride ($NiCl_2.6H_2O$), cadmium chloride ($CdCl_2$), cobalt sulphate ($CoSO_4.7H_2O$), lead nitrate ($Pb(NO_3)_2$) and silver nitrate ($AgNO_3$). We used the cup method which includes boring a hole in a media containing lawn culture and placing metal solutions in the hole to observe growth around it. The concentrations of heavy metals used were based on the concentrations determined to be sensitive to *E. coli* [8, 9].

Sensitivity to antibiotics

Antibiotic sensitivity to the arsenic resistant bacteria was determined by the standard Muller Hilton disc-agar diffusion test. Antibiotic saturated disc of 6 mm



(a) (b)

(c)

Figure 3. Growth of bacteria in nutrient agar supplemented with arsenite and arsenate for determination of MTC. As seen in the agar plates, growth stops at higher concentrations while being unhindered at lower concentrations due to resistance. *E. coli* grows at 3 Mm (a) and 10 Mm arsenite concentration (b), but it is unable to grow at high concentrations (40 mM) of arsenite and arsenate (c).

Table 2. The concentrations of heavy metals used based on the concentrations determined to be sensitive to *E. coli* [8, 9]. The WHO limit for drinking water and daily intake limit are also given [15, 16].

Heavy Metal	*Molarity (mM) used for assay*	*Concentration (mg/L) for assay*	*WHO guideline drinking water limit (mg/L)*	*Tolerable intake to humans (daily)*
$ZnCl_2$	1	136.31	3	1 mg/kg
$CuSO_4$	1	249.7	2	3 mg
$HgCl_2$	0.01	2.72	0.006	2 µg/kg
$NiCl_2$	1	237.69	0.07	12 µg/kg
$CdCl_2$	0.5	91.66	0.03	7 µg/kg
$CoSO_4$	1	281.1	-	-
$Pb(NO_3)_2$	5	1656	0.01	25 µg/kg
$AgNO_3$	0.002	3.39	-	7 µg/kg

Note: Data not available for cobalt and silver due to lack of literature according to WHO.

diameter (Hi media) were placed on Muller Hilton agar previously swabbed with bacterial cell suspension and incubated at 37°C for 24 hours. The diameters of the inhibition zones were noted and interpretations of the zones were made on the basis of the chart given by the manufacturer. (Hi Media) The antibiotics used were Ciprofloxacin (CIP30, 30 µg), Nalidixic Acid (NA30, 30 µg) and Gentamicin (GEN10, 10 µg).

Screening for As (III) oxidation and As (V) reduction activity

The bacteria were tested on the ability to oxidize As (III) or reduce As (V) using a qualitative $AgNO_3$ screening method [10, 11]. The bacteria were grown on nutrient broth with either 129.91 mg/L $NaAsO_2$ (sodium arsenite) or 312.01 mg/L Na_2HAsO_4 (sodium arsenate). After forty eight hours an mL of the culture was pipetted out and centrifuged at 5000 G for 15 minutes in triplicates. The collected pellet was washed with distilled water twice then suspended on 20 µl of distilled water and 80 µL of Tris HCl buffer (at pH 7.4). An addition of 0.1 µL of 1M arsenate or arsenite was made to make the final concentration 1 mM of arsenic. This is now incubated for 48 hours at 30°C. After the incubation period, 100 µL of 0.2 M $AgNO_3$ was added and any color changes noted. A light brown color in mixtures containing arsenite indicates positive arsenite reduction reaction (reduction of the arsenite in the mixture to arsenate). A light yellow color in mixtures containing arsenate indicates positive arsenate oxidation reaction (oxidation of the arsenate in the mixture to arsenite). The resulting precipitate is due to the formation of Ag_3AsO_3 (silver orthoarsenite, yellow) and Ag_3AsO_4 (silver orthoarsenate, brown-red).

RESULTS AND DISCUSSION

Arsenic is a very highly toxic metalloid which is responsible for a worldwide health problem especially in developing countries. Due to increasing contamination of soil, water and crops by arsenic, it has become a global problem [12]. Trivalent arsenite and pentavalent arsenate collectively known as inorganic arsenic are the most prevalent forms of arsenic in the environment. It is known that inorganic arsenic is highly toxic and among those arsenite is most toxic [13, 14]. Arsenate acts as a phosphate analogue and therefore disrupts metabolic reactions and enzymes that include phosphate while arsenite directly disrupts enzymatic function and structure.

As we know *E. coli* is a model organism. A lot of research has been aimed towards *E. coli* with lot of interest from fields like molecular biology. Genetic modifications and transformations are easier and well accounted for in *E. coli*. From this study we intend to

isolate and analyze an arsenic resistant *E. coli* capable of biotransformation. This *E. coli* will be able to grow in conditions with high arsenic concentrations which can have enormous use in bioremediation.

Determination of maximum tolerance concentration (MTC)

Determination of MTC is done by subsequent subculture in presence of gradually increasing concentration of arsenite and arsenate. The resistance of bacteria was tested in both nutrient agar and nutrient broth supplemented with sodium arsenite and sodium arsenate. The following Table 1 shows the resistance profile of *E. coli*.

Determination of heavy metal resistance

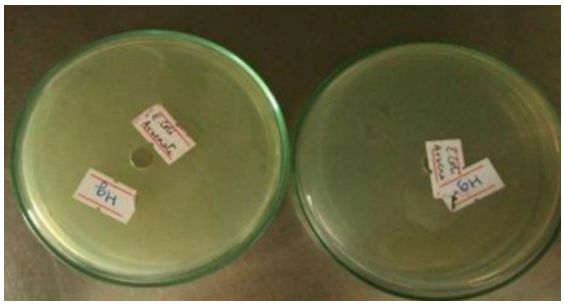
The bacteria were also studied for resistance to heavy metals other than arsenic. Resistances to other heavy metals were determined for zinc chloride ($ZnCl_2$), copper sulphate ($CuSO_4.5H_2O$), mercuric chloride ($HgCl_2$), nickel chloride ($NiCl_2.6H_2O$), cadmium chloride ($CdCl_2$), cobalt sulphate ($CoSO_4.7H_2O$), lead nitrate ($Pb(NO_3)_2$) and silver nitrate ($AgNO_3$). We used the cup method which includes boring a hole in a media containing lawn culture and placing metal solutions in the hole to observe growth around it. We saw that *E. coli* was resistant to all except lead nitrate.

Sensitivity to antibiotics

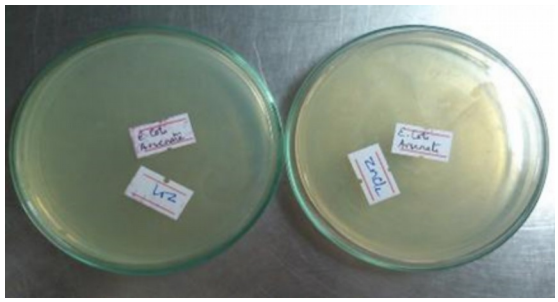
Antibiotic sensitivity to the arsenic resistant bacteria was determined by the standard Muller Hilton disc-agar diffusion test. Antibiotic saturated disc of 6mm diameter (Hi media) were placed on Muller Hilton agar previously swabbed with bacterial cell suspension and incubated at 37°C for 24 hours. The diameters of the inhibition zones were noted and interpretations of the zones were made on the basis of the chart given by the manufacturer. (Hi Media) The antibiotics used were Ciprofloxacin (CIP^{30}, 30 µg), Nalidixic Acid (NA^{30}, 30 µg) and Gentamicin (GEN^{10}, 10 µg).

According to Zone Size Interpretative Chart (Based on Results obtained using Mueller Hinton Agar) from HIMEDIA, *E. coli* grown exclusively in arsenate and arsenite is sensitive to all the above antibiotics [16, 17].

Here we can see that the *E. coli* is sensitive to multiple antibiotics. In bioremediation we tend not to use organisms that have multiple drug resistance. Therefore we can say from this particular point of view this *E. coli* is optimal for bioremediation.



(a)



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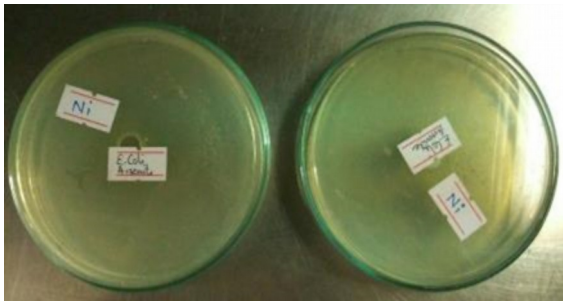
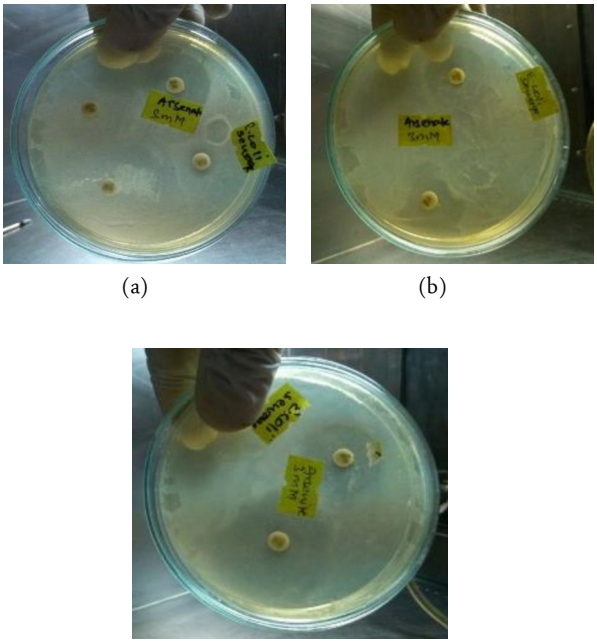


(c)

Figure 4. Heavy metal resistance assay. The cup method includes boring a hole in the middle of the recently inoculated agar plate (lawn culutre) and placing the metal solution inside the hole. The growth or lack thereof around the hole shows the extent of resistance the bacteria has on the metal solution. The concentration of metal solution used is deemed inhibitory for a standard *E. coli* [8, 9] (Arsenate/Arsenite on the tag indicate the *E. coli* in this culture originated from a plate supplemented with arsenite/arsenate). *E. coli* grow at 0.01 mM mercury concentration (a), 1 mM zinc concentration (b), and 1 mM nickel (c).

Table 3. Antibiotic resistance for bacteria

Antibiotic	*Concentration (mcg)*	*E.coli grown exclusively (mm)*	
		Arsenate	*Arsenite*
Ciprofloxacin	30	40	32
Gentamicin	10	24	27
Nalidixic Acid	30	25	22



(a) (b)

(c)

Figure 5. Mueller-Hinton disc-agar diffusion test for antibiotic sensitivity. Discs soaked in particular antibiotic is placed in a recently inoculated MH agar plate. The size of the non-growth halo regions around the disc shows effectiveness of the antibiotic to the bacteria. Inhibitory zone in antibiotic sensitivity test of *E. coli* grown exclusively in arsenate (a and b) and in arsenite (c).

Screening for As (III) oxidation and As (V) reduction activity

The bacteria were tested on the ability to oxidize As (III) or reduce As (V) using a qualitative $AgNO_3$ screening method [10, 11]. A light yellow color in mixtures containing arsenate indicates positive arsenate oxidation reaction (oxidation of the arsenate in the mixture to arsenite). The resulting precipitate is due to the formation of Ag_3AsO_3 (silver orthoarsenite, yellow) and Ag_3AsO_4 (silver orthoarsenate, brown-red). *E. coli* showed a light yellow color. This means that the arsenate supplied was transformed into arsenite. Although absence of any brown coloring shows that arsenite was not transformed to arsenate [10, 11].

Escherichia coli is a gram negative, rod shaped bacteria which is normal inhabitant of the lower gastrointestinal tract of warm blooded animals. *E. coli* has rare ability of utilization of lactose which is found in sugar of milk only found in mammals. In addition to thriving in the GI tract, *E. coli* has ability to survive outside the body. Environmental *E. coli* can migrate through feces of mammals and survive in extremely harsh and variable conditions as cold environment and trace nutrients. These two habitats are about as opposite to each other.

E. coli is a single-celled organism that can be manipulated and killed with no ethical concerns. It has a rapid growth rate and is very easy to culture in laboratory. *E. coli* can survive in uneven growth conditions including uneven temperatures, oxygen content, and nutrient availability. Most strains of *E. coli* are harmless, posing no threat to the scientists that use them. *E. coli* genetics are well-studied and can be manipulated easily [18]. In addition the *E. coli* we have isolated is arsenic resistant therefore it has a lot of potential with respect to molecular cloning and bioremediation.

Arsenic is very toxic to large number of microorganism but some have known to evolve features or defense mechanism which allows it to survive its presence. These mechanisms usually expel arsenic in one form or the other after it enters the cell. From the data available we can conclude this *E. coli,* can in fact detoxify it using redox changes between the species of arsenic. We have seen that this organism is able to alter the speciation of the medium around it as we have seen from biotransformation. *E. coli* is a model organism and has been topic of interest for many researchers from fields like molecular biology and recombinant DNA technology. Therefore an *E. coli* with a built-in arsenic resistant mechanism has a huge potential in any fields which includes growth in highly contaminated medium. Molecular technology like PCR and gene cloning has been highly optimized and simplified in *E. coli*, therefore it will be easier to add any gene into it. From the data we have seen this organism is sensitive to a number of antibiotics. In bioremediation one tends not to use an organism with multiple drug resistance to prevent any unforeseen hazardous situations. If this organism were to be used one would need a safeguard against it as *E. coli* can become virulent. This particular *E. coli* has high level of tolerance to arsenic which exceeds concentration in most waters naturally found.

As we know, *E. coli* is opportunistic pathogen whose sensitivity to antibiotics is an advantage. This *E. coli* also has shown resistance to many heavy metals which were considered sensitive to standard *E. coli*. Although this resistance may not be connected to arsenic resistance, it does show promise in similar application for heavy metals other than arsenic.

CONCLUSION

The ability of this organism to biologically transform arsenic, multiple heavy metals resistance and its stable growth in presence of highly toxic As (III) encourage future in-depth study to explore its role in arsenic mobilization and bioremediation. This particular *E. coli* has resistance to arsenic concentrations higher than naturally found. This can be useful for bioremediation and heavy metal recovery. The genome of *E. coli* is also extensively studied and gene transfer from this to another more preferable organism also has significant potential.

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