ISOLATION AND CHARACTERISATION OF ARSENIC RESISTANT BACTERIA FROM SOIL

Report

Submitted by

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To

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CERTIFICATE

This is to certify that the thesis entitled "ISOLATION AND CHARACTERISATION OF ARSENIC RESISTANT BACTERIA FROM SOIL". Embodies the work carried out by SOFI JEELANI under my supervision and this work is worthy of consideration for the award of M.Tech in Biotechnology.

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DECLARATION

I hereby affirm that the work entitled study to the arsenic resistant bacteria isolation presented in this thesis is exclusively my own work carried out at L.P.U. It does not contain any work for which a degree/diploma has been awarded by any other University/ Institute.

SOFI JEELANI

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I would like to express the deepest appreciation to my supervisor for all I have learned from him and who has shown the attitude and the substance of a genius: he continually and persuasively conveyed a spirit of adventure in regard to research and helping us to shape our interest and ideas, and an excitement in regard to teaching. Without his supervision and constant help this pre-dissertation project would not have been possible.

I would like to thank all the members of lovely school of biotechnology for giving us the permission to commence this project to do the necessary project work. I would like to thanks my parents for their unconditional support, both financially and emotionally throughout my project.

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CONTENTS

CHAPTER :1

INTRODUCTION

Economical and industrial activities increasing along with population growth contribute to spreading of various environmental contaminants.Carcinogenic, mutagenic and teratogenic arsenic (As) is a major constituent in more than 245 minerals and is ubiquous in the environment. Arsenic is a known carcinogenic that causes skin lesions, acute and chronic venenation, skin, liver and lung cancer and more over it is associated with diabetes, hypertension and capillary malady. Both acute and chronic poisoning to humans has raised great concerns, especially in heavily contaminated areas such as Bangladesh and West Bengal, India. The serious health problems were described as "the greatest mass poisoning in human history" by World Health Organization (Vaughan, 2006).

It is widely distributed in the nature. Arsenic can exist in oxidation states: Arsenate As(V), Arsenite As(III), elemental As(0) and Arsenide As(-III). As(V) is the less mobile form, chiefly because it is strongly adsorbed to the surface of several common minerals, such as ferrihydrite and alumina, whereas As(III) is more mobile as it adsorbs less strongly. Arsenic is one of the heavy metals that have poisonous effects on humans, plants, animals and also microorganisms. Arsenic concentration usually varies from below ten mg/kg in non-contaminated soil to as high as 30,000 mg/kg in contaminated soil. Pollution of soils with heavy metals is becoming of the most extreme environmental and human health hazards. Elevated levels of heavy metals not only decrease soil microbial activity and crop production, but also threaten human health through the food chain.

The bacterial resistance with regard to reduction of arsenate or oxidization of arsenite can be divided into two basic categories consisting of either detoxification reactions or redox reactions.The trivalent arsenite [+3, As (III)] can be converted to 100 times less toxic pentavalent arsenate [+5, As (V)] by the process of oxidation. Thus the oxidation of arsenite to arsenate is considered as primary method of detoxification. This oxidation process can be achieved by using chemical as well as biological methods. The chemical methods are costly and result in secondary pollution. Hence, alternatively biological or bioremediation methods of arsenite remediation are preferred. The most widespread resistance mechanisms uncovering in bacterium are ars operon which are either chromosomally or plasmid encoded. The two most common types of these operons contain either five (arsRDABC) or three (arsRBC) genes . Both Gram-negative and Gram-positive bacteria have been breakthrough out to use the same biochemical mechanism, encoded by basically the same plasmid or chromosome genes, nevertheless that the issue of genes can vary and the details of their role differ . The arsenic resistant antigenic determinant (ars), isolated from both Gram-positive and Gram-negative bacteria have been found to consist of either three or five genes, which have been organized into a single transcriptional unit. Several remediation techniques for arsenic removal have been applied, e.g., ion telephone exchange , adsorption with activated alumina and activated carbon, ultrafiltration, reverse osmosis, and

complexation with alloy ions followed by coagulation. These methods required large amounts of chemical reagents, for example, adsorption and ion exchange are expensive when use in the event of high concentration of heavy metal ions. The health hazards associated with arsenic-contaminated soil coupled with the high remediation cost makes it necessary to develop cost-effective technologies to restore polluted land.

CHAPTER 2:
Rationale of study

Arsenic contamination in the environment promotes the development of its detoxification by the use of arsenic reducing bacteria. Although arsenic concentration in soils of Punjab has been reported earlier, but understanding the introduction of arsenic in soil through agricultural pestcides, textile dyes,chemicals and mordants seem necessary. We planned the study of analyzing textile dyes contaminated soil for arsenic and other heavy metals and development of arsenic detoxification system in microorganisms present in soil.

CHAPTER 3:
OBJECTIVES AND SCOPE

The major objectives of the study are:

- To isolate the arsenic resistant bacteria from contaminated soil sample.
- To study the effect of arsenic on bacterial growth.
- Characterization of arsenic absorbing isolates.

Soil which is having the long term exposure to the arsenic contamination may result in the evolution of highly diverse arsenite-resistant bacteria. Bacteria capable of arsenite oxidation had an very high arsenite resistance level and hence can be used as potential candidate for bioremediation. Also, suitable in situ techniques may be developed for the isolated strain and there is potential of improvement in strain by the recent advancement in the field of genetic engineering.

Chapter-4

REVIEW OF LITERATURE

Bachate et al., (2008) have analysed the arsenic-resistant bacterial communities of two agricultural soils of Bangladesh. They isolated arsenic-resistant bacteria, to study their potential role in arsenic transformation and to investigate the genetic determinants for arsenic resistance among the isolates. Enrichment cultures were performed in a minimal medium in the presence of Arsenite and Arsenate to isolate resistant bacteria. Twenty-one arsenic-resistant bacteria belonging to different genera of Gram-positive and Gram-negative bacteria were isolated.They concluded that phylogenetically diverse arsenic-resistant bacteria present in agricultural soils of Bangladesh are capable of reducing arsenate to arsenite under aerobic conditions apparently for detoxification purpose.

This study provided results on identification, levels of arsenic resistance and reduction of arsenate by the bacterial isolates which could play an important role in arsenic cycling in the two arsenic-contaminated soils in Bangladesh.

Aksornchu et al., (2008) isolated arsenic-tolerant bacteria from contaminated soil collected in Ronphibun District, NakornSrithammaratProvince.Then they followed it by screening these bacteria for their ability to adsorb arsenite..Twenty-four bacterial isolates were obtained from samples cultivated in basal salts medium plus 0.1% yeast extract and up to 40 mM sodium-arsenite at 30oC under aerobic condition. From these, isolates B-2, B-3, B-4, B-21, B-25 and B-27 produced extracellular polymeric-like substances into the culture medium, which may potentially be used in the bioremediation of arsenic and other contaminants. All isolates displayed arsenite adsorbing activities in the ranges of 36.87-96.93% adsorption from initial concentration of 40 mM sodium-arsenite, without any arsenic transforming activity. Five isolates with the highest arsenite adsorbing capacity include B-4, B-7, B-8, B-10 and B-13 which adsorbed 80.90, 86.72, 87.08, 84.36 and 96.93% arsenite, respectively. Identification of their 16S rDNA sequences showed B-7, B-8, and B-10 to have 97%, 99% and 97% identities to *Microbacteriumoxydans, Achromobactersp.* and*Ochrobactrumanthropi*, respectively. Isolates B-4 and B-13, which did not show sequence similarity to any bacterial species, may be assigned based on their morphological and biochemical characteristics to the genus *Streptococcus*and *Xanthomonas,* respectively.

Thus, both isolates B-4 and B-13 appear to be novel arsenite adsorbing bacteria within these genus.

Bhosale et al., (2014) haveisolated three arsenic reducing bacterial strains designated as AR_2, AR_4 and AR_{10} from the waste of electroplating industry. The strains were identified by 16S rDNA sequencing and were found to be *Bacillus oceanosediminis*(AR_2) and *Achromobacterpulmonis*(AR_4 and AR_{10}). All the strains resisted both As(III) and As(V), common inorganic species of arsenic found in nature. Strains reduced As(V) to As(III) aerobically thus suggesting the presence of arsenic detoxification mechanism coded by arsoperon. Strains AR_4 and AR_{10} which belonged to *Achromobacter*genus did not show As(III) oxidation, unlike previously reported *Achromobacter*species, thus suggesting that arsenic transformation capabilities may differ among different species of same genus.

Krumova et al., (2008) isolated *Pseudomonas putida*K6 and *Pseudomonas mendosina*M3, which were of particular interest, because they appeared to gain metabolic energy during the arsenic transformation, were characterized. After screening test for genes from aox and ars – operon, it was detected only the gene for arsH protein in strains as *Ps. Stutzeri* and *Ps. mendosina*. For the first time, in bacteria identified as: *Ps.Stutzeri*and*Ps. mendocina,* was sequenced the gene for arsenic resistant protein - arsH, which shows high similarity with the same gene in *Ps. fluorescens*and *Ps. putida.*It is possible the genes for the arsenic-transforming enzymes in the investigated bacteria to be very different from the known gene encoded the same enzymes in the related bacteria.

Because of the toxic effect of arsenic in environment, all these investigation represent good basis for development of bioremediation technologies .

Srivastava et al., (2012) did a research in which arsenic contaminated rhizospheric soils of West Bengal, India were sampled for arsenic resistant bacteria that could transform different arsenic forms. *Staphylococcus sp.* NBRIEAG-8 was identified by16S rDNAribotyping, which was capable of growing at 30,000 mg/l arsenate and 1,500 mg/l arsenite. This bacterial strain was also characterized for arsenical resistance (*ars*) genes which may be associated with the high-level resistance in the ecosystems of As-contaminated areas. A comparative proteome analysis was conducted with this strain treated with 1,000 mg/l As(V) to identify changes in their protein expression profiles. A 2D gel analysis showed a significant difference in the proteome of arsenic treated and untreated bacterial culture. The change in pH of cultivating growth medium, bacterial growth pattern (kinetics), and uptake of arsenic were also evaluated. After 72 h of incubation, the strain was capable of removing arsenic from the culture medium amended with arsenate and arsenite [12% from As(V) and 9% from As(III)]. The rate of biovolatilization of As(V) was 23% while As(III) was 26%, which was determined indirectly by estimating the sum of arsenic content in bacterial biomass and medium. This study demonstrates that the isolated strain, *Staphylococcus sp.*, is capable for uptake and volatilization of arsenic by expressing *ars* genes and 8 new upregulated proteins which may have played an important role in reducing arsenic toxicity in bacterial cells and can be used in arsenic bioremediation.

Xiong et al., (2006) isolated 32 arsenic resistant bacterial strains from the mining wastes of Shangrao coal mine of China. Twelve were isolated using arsenite enrichment cultivation method and twenty were identified using the culture-independent 16S rDNA library technique. Analysis of 16S rDNA revealed that the belong to 4 different phylogenetic Clades. They are *Rhodococcus, Arthrobacter, Cupriaridus, Acinetobacter, Pseudomonas, Agrobacterium,Sinorhizobium, Bradyrhizobium, Pseudomonas, Rhodobium, Bacillus, Clostridium*and some uncultured environmental clones. Strain C13 showed the highest arsenite resistant level. This strain was able to oxidize arsenite to arsenate aerobically and was identified as Agrobacterium sp.. Another two strains, C8 and C14, were able to reduce arsenate to arsenite and were identified as Arthrobacter sp. and Cupriavidus sp., respectively.

Pepi et al., (2006) isolated arsenic-resistant bacteria from contaminated sediment of the Orbetello Lagoon, Italy, to characterize isolates for As(III), As(V), heavy metals resistance, and from the phylogenetic point of view.From their study they concluded that isolates are highly resistant to both As(III) and As(V) and they could represent good candidates for bioremediation processes of native polluted sediments.
This study provides original results on levels of resistance to arsenic and to assigning genera of bacterial strains isolated from arsenic-polluted sediments.

Nagvenkar and Ramaiah (2010) in their research removed the bacterial isolates from water and sediment samples from freshwater, estuarine and marine regions and were tested for their growth in the presence of different concentrations of arsenic. Despite the generation times being longer in case of all bacterial isolates tested in nutrient broth with 200ppm Arsenite (As3+), many of them were able to attain log phase and substantial growth variously between 36 and 96 hours. The isolates tolerating ≥ 200 ppm arsenic (As) were found to belong to *Enterobacteriaceae, Pseudomonas, Corynebaterium, Xanthomonas, Acinetobacter, Flavimonas*and *Micrococcus*. The percent of arsenite biotransformed/removed from the growth medium was the highest by a strain of Enterobacteriaceae (as much as 92% of the As in the growth medium by 120hrs) followed by that of Corynebaterium and Acinetobacter strains. From these observations it is clear that many environmental strains are capable of quite rapid biotransformation of As. Contamination of drinking water by toxic metalloid arsenic affects
thousands of people worldwide.

Huang et al., (2006) did their research under the heading "Characterization of arsenic-resistant bacteria from the rhizosphere of arsenic hyperaccumulator Pteris vittata". From their work they found thet arsenic hyperaccumulator fern Pteris vittata L. produces large amounts of root exudates that are hypothesized to solubilize arsenic and maintain a unique rhizosphere microbial community. Total heterotrophic counts on rich or defined media supplemented with up to 400 mmol/L of arsenate showed a diverse arsenate-resistant microbial community from the rhizosphere of P. vittata growing in arsenic-contaminated sites. Twelve bacterial isolates tolerating 400 mmol/L of arsenate in liquid culture were identified. Selected bacterial isolates belonging to different genera were tested for their resistance to osmotic and oxidative stresses. Results showed that growth was generally better under osmotic stress generated by arsenic than under that generated by NaCl or PEG 6000, demonstrating that arsenic detoxification metabolism also cross protected bacterial isolates from arsenic-induced osmotic stress.

Moghadami and Payeghadr (2013) did a study in payam noor university in Tehran and the aim of their study was to isolate native arsenic bacteria from gold mine and then evaluate their tolerance. In their study four strains of bacteria were isolated from the soil of Takab gold mine. These isolates were identified at genus level.They belonged to these genera: *Bacillus, Staphilococcus, Dienococcus and Roseomonas*.The later one was able to grow in 2 ppm of arsenic. The results of this study showed that Roseomonas strain could not tolerate up to 3 ppm of gold in the presence of 2 ppm arsenic, while when *Roseomonas* strain was cultured in the medium without arsenic it was able to grow in 3 ppm of gold. In the other word the amount of arsenic impress on the gold tolerance of bacteria. Isolation of bacteria and evaluation of using arsenic in them is ongoing because these bacteria can used to help remove arsenic pollution such as remove arsenic from water.

Kafilzadeh et al., (2013) did a study in which the indigenous arsenic resistant bacteria of Lake Maharloo were identified. In this research arsenic-resistant bacteria from water and sediments of Lake Maharloo were isolated and then their antibiotic resistance was evaluated.
Bacteria such as *Bacillus sp, Vibrio sp, Staphylococcus sp, Corynebacterium sp, Micrococcus sp, Pseudomonas sp* and *E .coli* were isolated in different seasons from water and sediments of Lake Maharloo. The maximum and minimum abundance percentage of arsenic-resistant bacteria was found in sediments of spring (56.25%) and winter (12.50%) ($P<0.05$) respectively.
Antibiotics resistance patterns evaluation revealed that isolated bacteria had the most antibiotic resistance to penicillin and the lowest to amikacin in all seasons.

Liao et al., (2010) did a study on arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan.
The objective of this study was to investigate the relevant arsenite-oxidizing and arsenate-reducing microbial community that exists in highly arsenic-contaminated groundwater
in Taiwan. They cultured and identified arsenic-transforming bacteria, analyzed arsenic resistance and transformation, and determined the presence of genetic markers for arsenic

transformation. In total, 11 arsenic-transforming bacterial strains with different colony morphologies and varying arsenic transformation abilities were isolated, including 10 facultative anaerobic arsenate-reducing bacteria and one strictly aerobic arsenite-oxidizing bacterium. All of the isolates exhibited high levels of arsenic resistance with minimum inhibitory concentrations of arsenic ranging from 2 to 200 mM. Strain AR-11 was able to rapidly oxidize arsenite to arsenate at concentrations relevant to environmental groundwater samples without the addition of any electron donors or acceptors.In this study, they provided the first look at the relevant microbial community that exists in an arsenic-contaminated aquifer that is historically linked to extreme arsenic toxicity in Taiwan.

Titah et al., (2011) did a study on isolation and screening of arsenic resistant rhizobacteria of Ludwigia octovalvis.In their study a total of 109 epiphyte rhizobacteria were isolated from roots of L. octovalvis at five different arsenic concentrations which later could be characterized to 29 groups. After secondary screening, 12 rhizobacterial isolate were potentially resistant to arsenate. One of them was identified as *A. globiformis*which possibly used arsenic in their growth mechanism. The k value for *A. globiformis*was 0.112 h-1. MIC of arsenate exposure for *A.globiformis*was determined to be 500 mg/L.

Saltikov et al., (2001) studied the homology of E.Coli R773 at University of calfornia.Genes arsA, arsB, and arsC in arsenic-resistant bacteria were Isolated from raw sewage and arsenic-enriched creek waters. The occurrence and diversity of the Escherichia coli R773 ars operon were investigated among arsenic resistant enteric and nonenteric bacteria isolated from raw sewage and arsenic-enriched creek waters. Selected isolates from each creek location were screened for ars genes by colony hybridization and PCR. The occurrence of arsA, arsB, and arsC determined by low-stringency colony hybridization (31 to 53% estimated mismatch) was 81, 87, and 86%, respectively, for 84 bacteria isolated on arsenate and arsenite-amended media from three locations. At moderate stringency (21 to 36% estimated mismatch), the occurrence decreased to 42, 56, and 63% for arsA, arsB, and arsC, respectively. PCR results showed that the ars operon is conserved in some enteric bacteria isolated from creek waters and raw sewage. The occurrence of the arsBC genotype was about 50% in raw sewage enteric bacteria, while arsA was detected in only 9.4% of the isolates.The arsABC and arsBC genotypes occurred more frequently in enteric bacteria isolated from creek samples: 71.4 and 85.7% ,respectively. Average sequence divergence within arsB for six creek enteric bacteria was 20% compared to that of the E. coli, R773 ars operon. Only 1 of 11 pseudomonads screened by PCR was positive for arsB. The results from this study suggest that significant divergence has occurred in the ars operon among As-resistant E. coli strains and in *Pseudomonas spp*.

Kaushik et al., (2012) did a study on Arsenic Hyper-tolerance in Four *Microbacterium* Species Isolated from Soil Contaminated with Textile Effluent. Arsenic-contaminated areas of Sanganer, Jaipur, Rajasthan, India were surveyed for the presence of metal resistant bacteria contaminated with textile effluent. Samples were collected from soil receiving regular effluent from the textile industries located at Sanganer area. In total, nine bacterial strains were isolated which exhibited minimum inhibitory concentration (MIC) of arsenic ranging between 23.09 and 69.2mM. Four out of nine arsenic contaminated soil samples exhibited the presence of arsenite hyper-tolerant bacteria. Four high arsenite tolerant bacteria were characterized by 16S rDNA gene sequencing which revealed their similarity to *Microbacteriumparaoxydans strain 3109, Microbacterium paraoxydans strain CF36, Microbacterium sp. CQ0110Y, Microbacterium sp. GE1017*. These results indicate that the arsenic polluted soil harbors arsenite hyper-tolerant bacteria like *Microbacterium*which might play a role in bioremediation of the soil.

Majumder et al., (2012)in their study isolated twenty six Arsenic resistant bacterial stains from Arsenic(AS) contaminated paddy soil of West Bengal,India. Among them 10 isolates exhibited higher AS resistance capacity and could grow in concentration of 12000 mg/lt of arsenate and 2000mg/lt of arsenite in growing medium. Maximum growth was observed at 1000mg/lt and 100mg/lt in case of arsenate and arsenite respectively. Results of incubation study carried out in basal salt minimal media (BSMY) containing 25mg/lt of arsenate and arsenite separately showed that the isolates could accumulate 1.03-6.41 mg/lt of arsenate and 2.0 – 7.6 mg/lt of arsenite from the media.

Ghodsi et al., (2001)studied the bioremediation of arsenic by bacteria isolated from contaminated soil.The aim of their study was isolating arsenite-resistant bacteria from arsenic contaminated soil and the investigation of arsenite bioremediation efficiency by the most resistant isolates. Isolation of arsenite-resistant bacteria and the minimum inhibitory concentration (MIC) were conducted by spread plate method and the agar dilution method on PHG-II agar plates supplemented with sodium arsenite respectively. The results showed that, 69 and 25% of arsenite resistant isolates were gram positive and negative bacilli, respectively. Its maximum MIC was 128 mM/L, which is related to such bacteria as *Bacillus macerans, Bacillus megaterimand Corynebacterium vitarumen.*
Their results express the probability of finding more arsenic accumulating bacteria from the contaminated soil environment and can be concluded that arsenic resistant and/or accumulating bacteria, such as Bacillus sp., are widespread in the polluted soils and are valuable candidates for bioremediation of arsenic contaminated ecosystems.

Ahsan et al., (2011) did a research on the arsenic adsorption by bacterial extracellular polymeric substances. The main objective of this work was to isolate arsenic resistant bacteria from contaminated soil, followed by screening for their ability to adsorb arsenic. Six bacterial isolates (S1 to S6) were obtained from arsenic contaminated soil samples and among these, five (S1, S2,

S3, S5 and S6) were characterized as bacillus and the rest one (S4) was cocci depending on shape. All the isolates except S6 produced extracellular polymeric substances (EPS) in the culture medium and displayed arsenic adsorbing activities demonstrated by adsorption of around 90% from initial concentration of 1 mg/L sodium arsenite. To clarify the role of EPS, they killed the bacteria that produced EPS and used these killed bacteria to see whether they could still adsorb arsenic or not. They found that they could adsorb arsenic similarly like that of EPS produced live bacterial isolates. From the observation they concluded that these isolates showed potentiality to adsorb arsenic and hence might be used for bioremediation of arsenic.

Jackson et al., (2005) studied the enumeration and characterization of culturable arsenate resistant bacteria in a large estuary.They concluded from their study that arsenic is a toxic element that exists in two major inorganic forms, arsenate and arsenite. A number of bacteria have been shown to resist arsenic exposure, and even more bacteria appear to possess the genes for arsenic resistance. In this study, the numbers of culturable arsenate-resistant bacteria present in water at three coastal sites in the Lake Pontchartrain estuary, Louisiana, was determined. Despite insignificant (less than 1.33 μM) levels of arsenic in this system, 20–50% of the viable count of bacteria showed appreciable arsenate resistance, suggesting that arsenic-resistant bacteria are common and widespread. A diverse array of arsenate-resistant isolates was obtained, with 16S rRNA sequence analysis indicating 37 different bacterial strains, representing six major bacterial groups. Many of these isolates were affiliated with groups of bacteria that have been poorly characterized in terms of arsenic resistance, such as the *Betaproteobacteria* or *Flavobacteria*. Some isolates were capable of tolerating very high (>100 mM) levels of arsenate, although arsenite resistance was generally much lower. The results suggest that arsenic-resistant bacteria are common, even in environments with insignificant arsenic contamination, and that many different groups of aquatic bacteria show appreciable arsenic resistance.

Anderson and Cook (2004) did the Isolation and Characterization of Arsenate-Reducing Bacteria from Arsenic-Contaminated Sites in New Zealand. In their study the two environmental sites in New Zealand were sampled (e.g., water and sediment) for bacterial isolates that could use either arsenite as an electron donor or arsenate as an electron acceptor under aerobic and anaerobic growth conditions, respectively. These two sites were subjected to widespread arsenic contamination from mine tailings generated from historic gold mining activities or from geothermal effluent. No bacteria were isolated from these sites that could utilize arsenite or arsenate under the respective growth conditions tested, but a number of chemoheterotrophic bacteria were isolated that could grow in the presence of high concentrations of arsenic species. In total, 17 morphologically distinct arsenic-resistant heterotrophic bacteria isolates were enriched from the sediment samples, and analysis of the 16S rRNA gene sequence of these bacteria revealed them to be members of the genera *Exiguobacterium, Aeromonas, Bacillus, Pseudomonas, Escherichia,* and *Acinetobacter.*

Escalante et al., (2010) isolated arsenic resistant bacteria from arsenic contaminated river in the Atacama desert (chile).In this study arsenic resistant bacteria were isolated from the sediments of an arsenic contaminated river. Bacterial isolation was done on the agar plate.Forty nine bacterial strains were isolated. 55% corresponded to the reducing bacteria,4% to oxidizing bacteria, 8% presented both activities and in 33% none activity was detected.

CHAPTER 5:

Equipments and Materials Used:

Equipments used	Name of company
Laminar Air Flow	Rescholar Equipment.
Autoclave	Rescholar Equipment.
Weighing Balance	Rescholar Equipment.
Incubator	York scientific industries ltd.
Hot Air Oven	Narang scientific Pvt. Ltd.
Refrigerator	Goodrej
Spectrophotometer	IFB Care
Conventional oven	IFB Care

List of chemicals and Media used:

Chemical Name	Company Name
LB Agar	Titan biotech Ltd
Arsenic Trioxide	Loba chemie
Sulphuric Acid	Avantor.
Potassium Iodate	Loba chemie
Carbon Tetrachloride	Loba chemie
Ethanol	Changshu yanguan
Nutrient broth	Loba chemie

CHAPTER 6:

Research Methodology:

6.1 Collection of the soil sample: The soil sample was collected from the malwa region in Punjab.The malwa region is considered to be the most arsenic contaminated area in the state of Punjab.The collection was done at a depth of 15cm from the above surface.

Figure 1: Soil collection

6.2 Determination of arsenic from standard curve: The preparation of the standard arsenic stock solution was prepared by dissolving 0.1732gm of arsenic trioxide in 100ml of water.Then the working standard solution was prepared with different concentrations of stock solution.

Ist working standard solution: 0.1ml of stock + 1ml of potassium iodate(2o min at 37C +1ml sulphuric acid (10 Min) + 1ml carbon tetrachloride + distilled water (to make 10ml). For the other working standard solution the amount of stock solution is changed from 0.1 to 2.4.

After this the O.D of all the working standard will be taken and the graph will be ploted for the same.

6.3 Isolation of Arsenite Resistant Bacteria: For isolation of arsenite tolerant bacteria from the contaminated soil samples serial dilution method was applied.The soil sample was serially diluted by dissolving 1mg of soil in 9ml of distilled water.This is marked as the 1x dilution. From 1x 1ml is taken and put in another test tube having 9ml of distilled water in it.The same is repeated till 1000x dilution rate is done.Then the LB media is prepared in two different compositions:

- LB media with arsenic.
- LB media without arsenic trioxide.

Then the petriplates and the media will be autoclaved at 15 Psi for 15 minutes.After this the inoculation of the soil sample is done on the LB agar plates.The plates will be having media with arsenic in one and the media without arsenic in another.The streaking of the soil sample will be done and the plates will be kept in the incubator for 24-48 hrs for bacteria to grow.
Once the growth on the arsenic plates will be there, then the concentration of the arsenic will be increased and the growth of the bacteria will be checked.This will be done till the pure colonies were obtained with repeated streaking.

6.4 Determination of Minimum Inhibitory Concentration: The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration that completely inhibits bacterial growth. MIC for purified bacterial strains against arsenite was determined in luria broth amended with different concentrations of sodium arsenite. Cell density was measured by measuring the culture turbidity using a spectrophotometer.

6.5 Identification by various chemical and biochemical tests: The isolated strain that will be showing the maximum MIC will be subjected to the various tests to study various parameters.These tests include:

- **Gram staining.**
- **Indole test.**
- **Catalase test.**
- **Gelatin hydrolysis test.**
- **Oxidase test.**
- **MRVP test.**
- **Citrate test.**

CHAPTER 7:
EXPECTED OUTCOME:

The research focused upon the isolation of arsenite tolerant bacteria from contaminated soil sample, followed by the characterization of the bacteria.Hence it is expected that the bacterial isolates will be showing the resistance to the arsenite at the higher arsenic concentrations.The characterization of the bacteria by various chemical and biochemical tests will tell us about the various bacterial strains present.These strains can be used for the detoxification of the arsenite and the bioremediation purposes.

CHAPTER 8:
PROPOSED WORKPLAN WITH TIMELINE:

MONTH	WORKDONE
1. February	Lab allotment was done first.Collection of chemicals and the other glassware was done.
2. March.	Due to lots of vacations in this month, the workdone was not quite sufficient.After soil dilution the growth of bacteria on the LB media with and without arsenic was done.
3. April.	Different biochemical tests were performed.

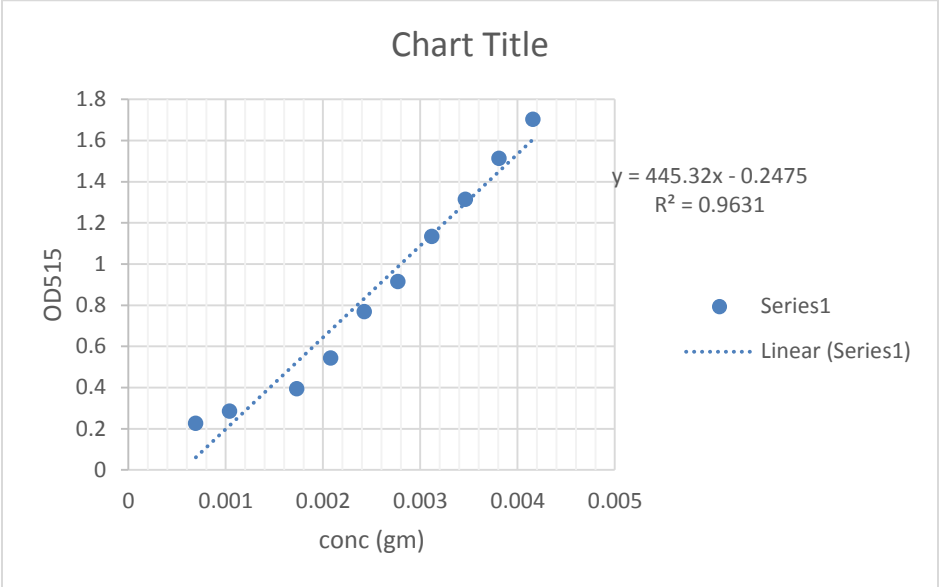
CHAPTER 9:
RESULTS AND DISCUSSION:

9.1 Soil sampling and its pH: The soil sample was collected from the agricultural land adjoining the sewage area from the malwa region. The physical and chemical properties of the soil samples were determined. The pH of the sample ranged from 7.27-8.61.

9.2 Determination of arsenic by standard curve: The standard curve was plotted by plotting a graph between the arsenic concentration VS optical density.The O.D was taken at 515nm. The graph plotted showed the increase in O.D value with the increase in the arsenic concentration.The arsenic concentrations and O.D values were as follows:

Arsenic conc.	O.D
0.000346	0.112
0.000693	0.226
0.001039	0.285
0.001732	0.393
0.002078	0.542
0.002425	0.767
0.002771	0.914
0.003118	1.132
0.003464	1.313
0.00381	1.512
0.004157	1.702

Figure 2: Different concentrations of standard working solutions.



9.3Isolation of Arsenite Tolerant Bacteria: The bacteria was grown first on the LB media without any arsenite in it.The bacterial colonies were visible.

Figure:3 LB agar without arsenic.

After this the bacteria was grown on the media having arsenite added to it.Here also the growth of bacteria was clear.The bacteria showed growth after 24 hrs incubation at 37$_{o}$C.

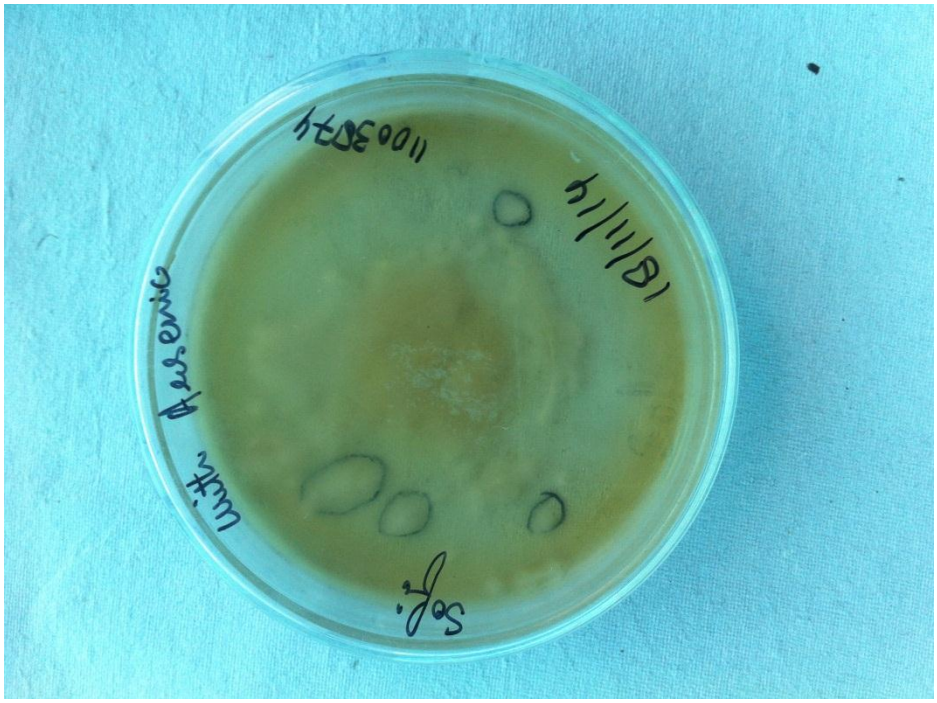


Figure 4: LB agar with arsenite

After the growth of bacteria on media having arsenic, the further plating was done by increasing the arsenite concentration from 1% to 8% and after this the MIC was observed because the bacteria stopped growing after this.

At 1% arsenic concentration i.e, 1mg arsenic trioxide in 100ml of LB media the bacteria was grown by plating the colonies of the earlier plate in which arsenic was already added and the growth was also there.

Figure 5: 1% arsenite concentration.

At 2% the bacteria also showed some growth but the colonies were not clearly visible.

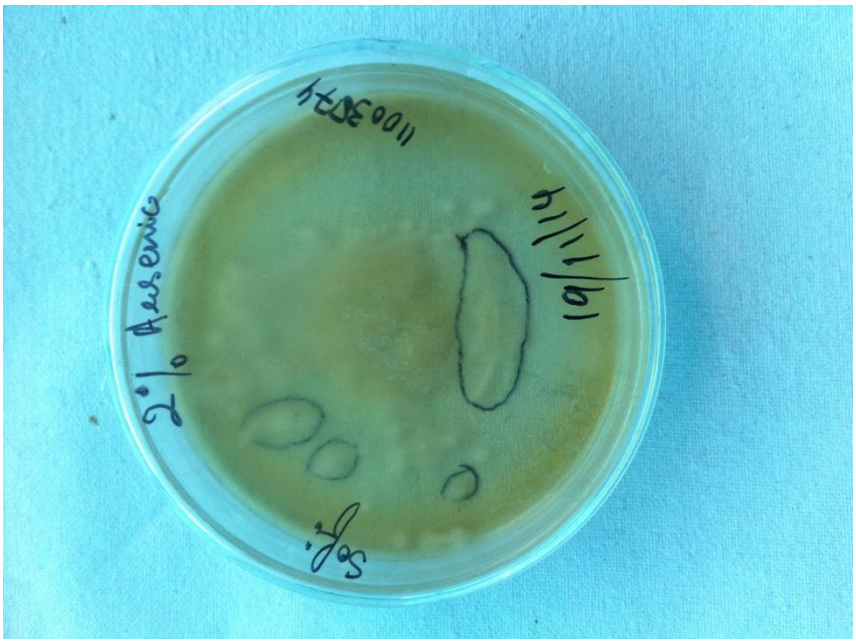


Figure 6: 2% arsenite concentration.

After this the concentration was increased to 4% and it gave the better colonies and colonies were clearly visible.

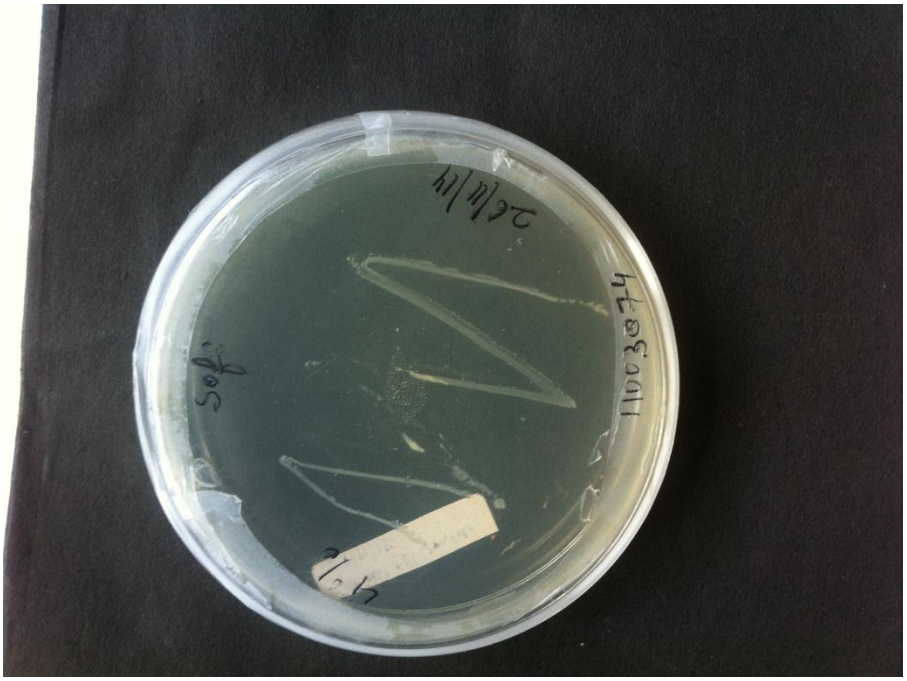


Figure 7: 4% arsenite concentration.

Again the concentration was increased to 6% and here also the colonies were very clear and the growth of the bacteria was illustrated.

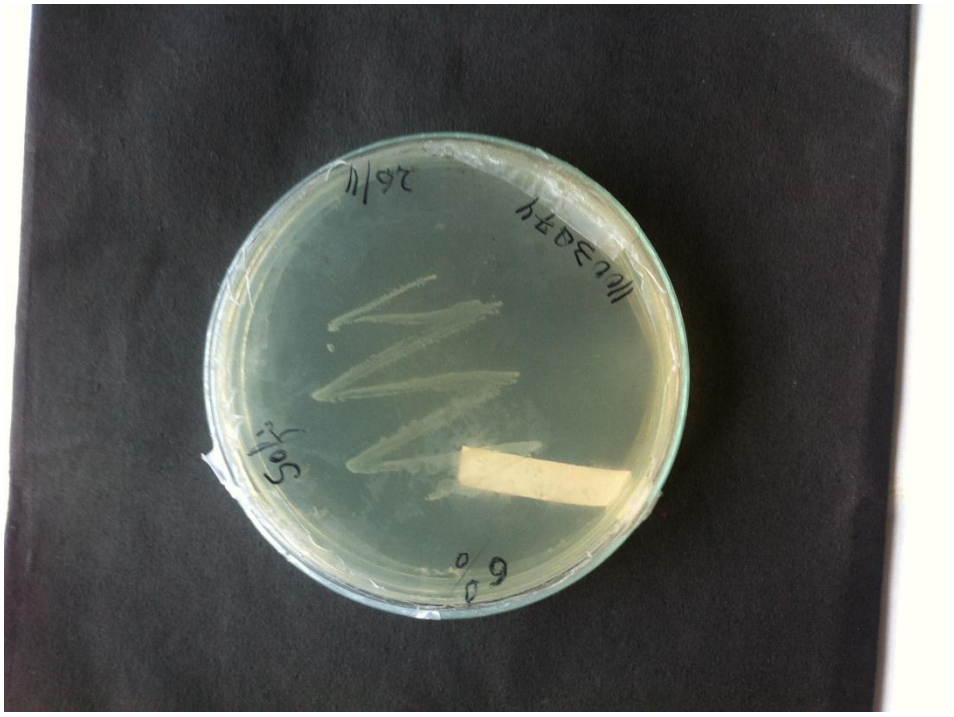


Figure 8: 6% arsenite concentration.

At the last the bacteria showed growth at 8% arsenite concentration and this showed that these bacterial strains are having resistance to the arsenite.Hence these can further be used for the characterizartion.

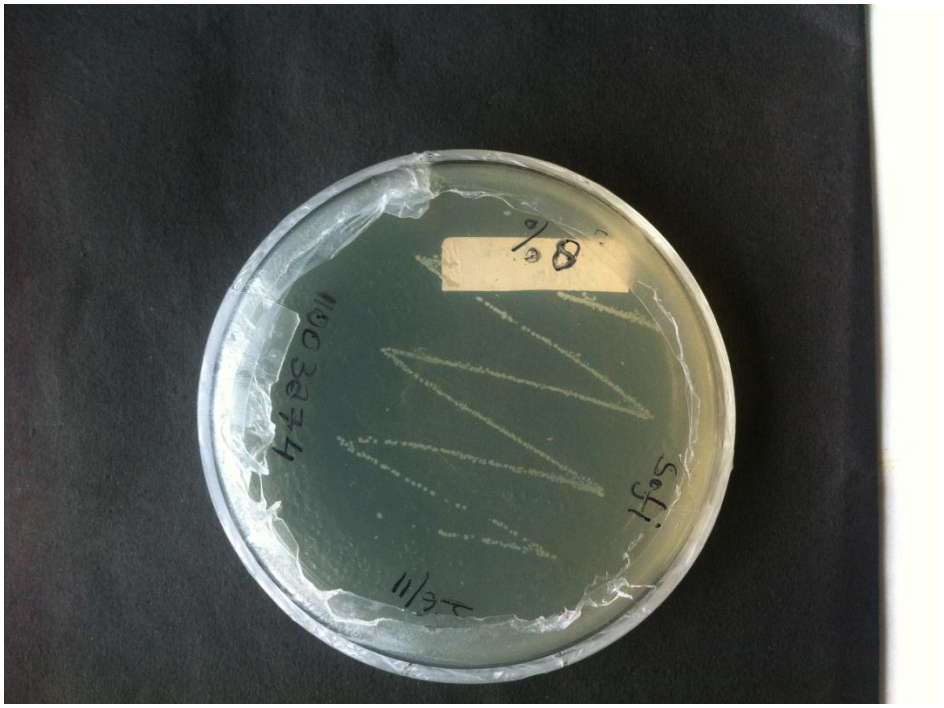


Figure 9: 8% arsenite concentration.

Biochemical Characterization:

1. **Gram staining:** This test is performed to differentiate between the two major categories of bacteria: Gram positive and Gram negative. The Gram stain is a very important preliminary step in the initial characterization and classification of bacteria.
   The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization step, a counterstain is used to impart a pink color to the decolorized gram-negative organisms.

   In our culture the strain was retained by the bacteria which showed that our bacteria is gram-positive. The bacterial culture cells were put on the slide and then the stain was added. The cells turned their color into purple, which showed that the bacteria isolated is gram-positive.

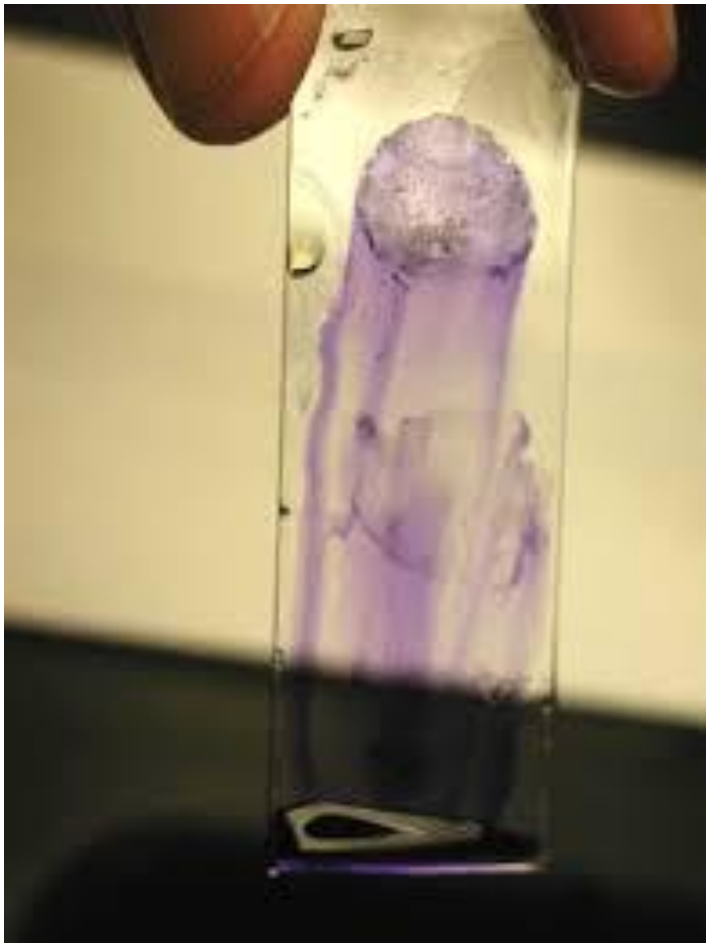


Figure 10 Purple color retained.

Indole test: Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indole.

Method:

a. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.
b. Incubate at 37°C for 24-28 hours in ambient air.
c. Add 0.5 ml of Kovac's reagent to the broth culture.

The results were negative in our case as there was no color change even after the addition of appropriate reagent.

Figure 11 No color change shows negative result.

Catalase test: Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme or not catalase test is performed.

Procedure of catalase test:

1. Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick
2. Place a drop of 3% H_2O_2 on to the slide and mix.
3. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.
4. A negative result is no bubbles or only a few scattered bubbles.

Result:

In our bacterial isolate the test showed positive results as the bubbling was there shortly after the addition of H_2O_2 .

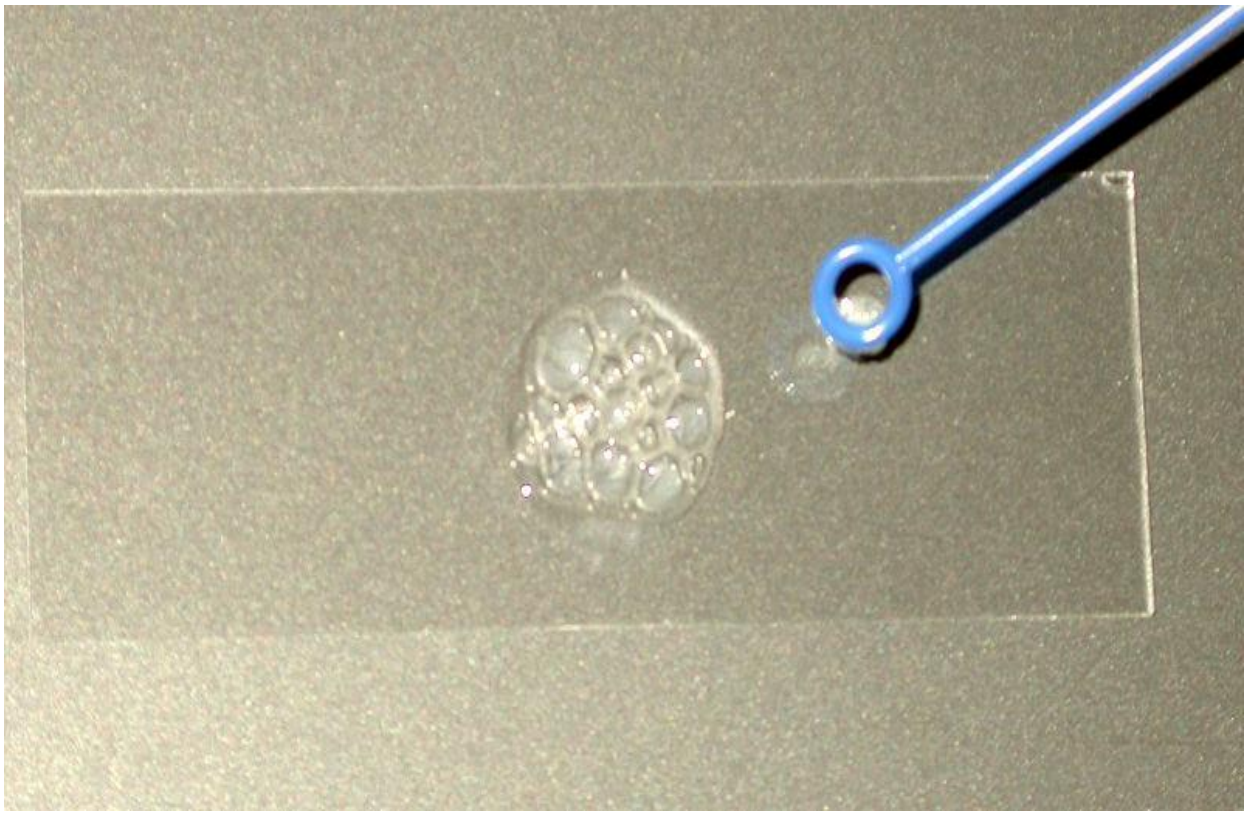


Figure 12 Bubbling showing release of oxygen.

Gelatin hydrolysis test: Gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy gelatin. Hydrolysis of gelatin indicates the presence of gelatinases.

Procedure:

1. Inoculate a heavy inoculum of test bacteria (18- to 24-hour-old) by stabbing 4-5 times (half inch) on the tube containing nutrient gelatin medium.
2. Incubate the inoculated tube along with an uninoculated medium at 35°C, or at the test bacterium's optimal growth temperature, for up to 2 weeks.
3. Remove the tubes daily from the incubator and place in ice bath or refrigerator (4°C) for 15-30 minutes (until control is gelled) every day to check for gelatin liquefaction.(Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath or kept in refrigerator at 4°C).
4. Tilt the tubes to observe if gelatin has been hydrolyzed.

Result: Partial or total liquefaction of the inoculated tube (uninoculated control medium must be completely solidified) even after exposure to cold temperature of ice bath or refrigerator (4°C). In our isolate the partial liquefaction was there even after exposure to cold temperature,hence the results were positive.

Figure 13 Left one is the one having bacterial isolate and the right one is control.

Oxidase test:

Procedure of Oxidase test:

1. Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride.
2. Then moisten the paper with sterile distilled water.
3. Pick the colony to be tested with loop and smear in the filter paper.
4. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

Result: The results were positive as the development of dark purple color (indophenols) within 10 seconds were visible.

Figure 14 clearly visible purple color.

MRVP Test: Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose.

Procedure for Methyl Red (MR) Test

MR-VP broth is used for both MR Test and VP test. Only the addition of reagent differs, and both tests are carried out consecutively.

1. Inoculate two tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.
2. Incubate at 35 °C for up to 4 days.
3. Add about 5 drops of the methyl red indicator solution to the first tube (for Voges-Proskauer test, Barrit's reagent is added to another tube).
4. A positive reaction is indicated, if the colour of the medium changes to red within a few minutes.

Result: MR Test positive- appearance of red color after the addition of methyl red reagent.

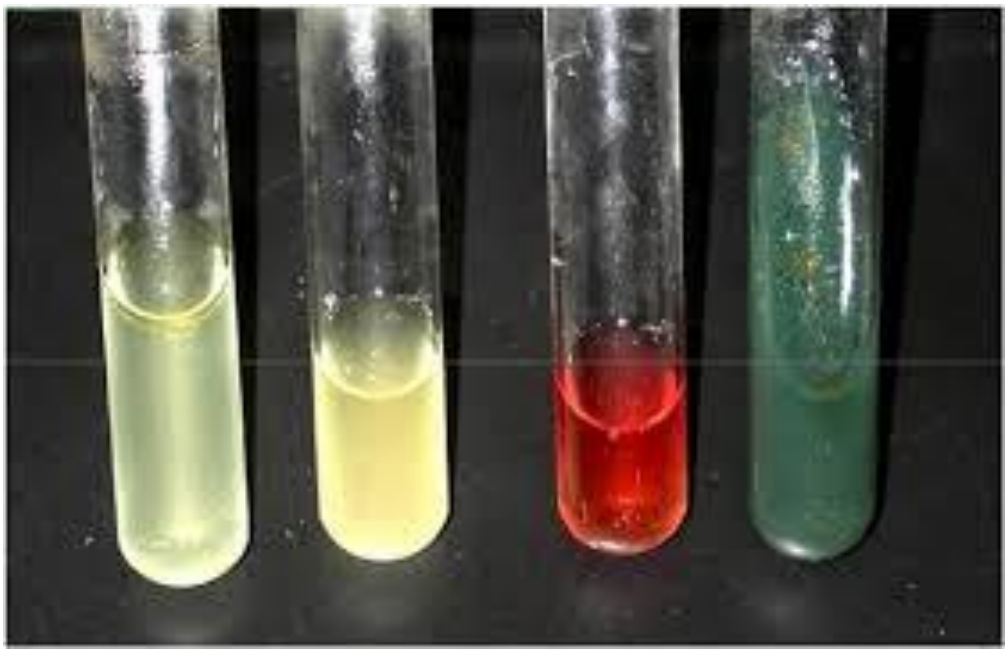


Figure 15 Red colored tube showed the positive reaction.

Citrate test:

Procedure of citrate utilization test:

1. Inoculate Simmons Citrate Agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
2. Incubate at 35^{o}C to 37^{o}C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
3. Observe the development of blue color; denoting alkalinization.

Result: Growth was visible on the slant surface and the medium had an intense Prussian blue.

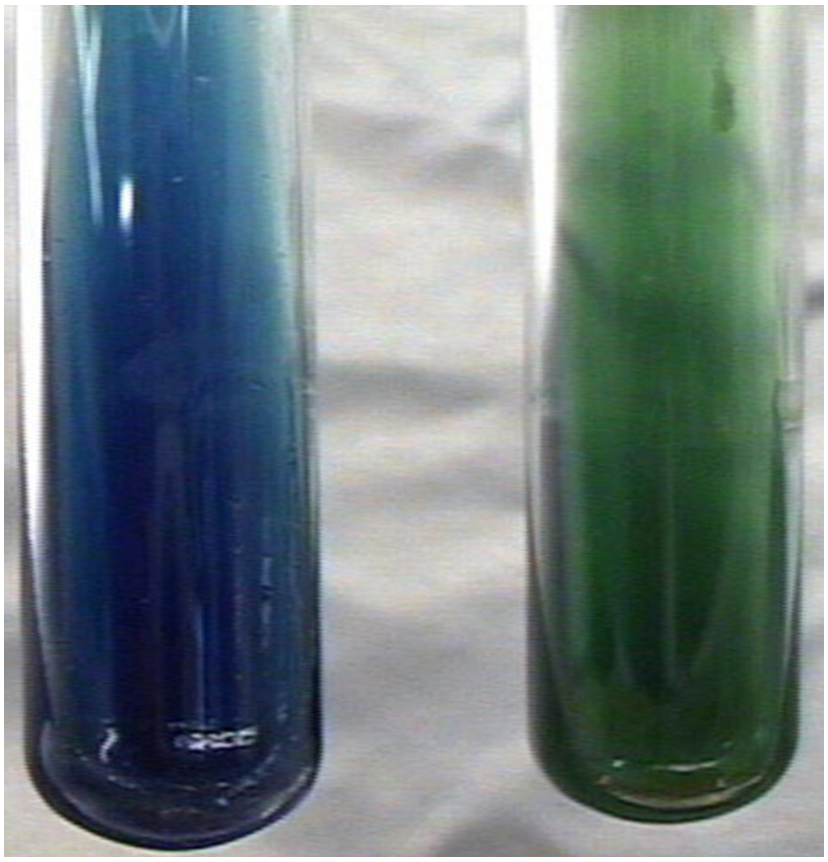


Figure 16 Blue color shows the positive result and the green one is the control.

CHAPTER 10:

CONCLUSION:

The soil samples from which the arsenite tolerant bacteria were isolated were contaminated with arsenic. The pH of the soil was neutral or alkaline.For the further analysis the chemical and biochemical characterization was done.The characterization was done by doing several tests on the isolated bacteria from the soil sample.

Biochemical characteristics of the As- resistant bacterial isolates has shown similarity to Klebsiella oxytoca and Citrobacter freundii when various results were compiled taken from the work that has been done by other people during the past course of time.

CHAPTER 11:
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