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Isolation and Molecular Identification of Arsenic Resistant Microorganisms Coming from Xichu River, Gto. Mexico.

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The arsenic is a toxic element for the human and other organisms´ health, it may affect the exploitation of water, the agriculture development and the sustainable rational use of soil, the result is reflected within the socioeconomic growth lag in the affected area. Arsenic is mobilized in the environment by a combination of several natural processes, such as weathering reactions, biological activities and volcanic emissions, as well as by a group of anthropogenic activities. Microorganisms interact with the arsenic through a different mechanism such as adsorption, redox, precipitation, etc. On the other hand, the rate of arsenic releasing in geochemical environments is controlled mainly by microorganisms. In the environment, the major arsenic transformations include microbial oxidation, reduction, methylation and demethylation, those reactions have an enormous impact in the arsenic behavior that depends on its redox state that exhibits differences in its mobility and toxicity. Normally, the arsenic resistance comes through the production of genes within operon ars, their location is usually plasmid or chromosomic. In this study, it was achieved the isolation of 11 strains from which 2 have been reported as an arsenic resistant in this work. The samples were collected from water, sediment and biofilm of Xichu River, which has been impacted by mining activity, exhibiting a high arsenic concentration (98 µg/l). All the strains were tested by several methods as arsenic resistance, colorimetric test for arsenate reduction, pDNA extraction as well as ars and aox genes amplification, which are the genes involved in arsenic transformation. It was obtained a great microbial diversity with different skills in arsenic transformation. One of the isolated strains Rodoccoccus gordoniae, did not show the presence of any gen but it was the most likely microorganisms to grow in the presence of arsenic. Almost all the microorganisms showed arsenic resistant until 20 mM of arsenite and arsenate. The main contribution of this study is the development of knowledge to understand much better the arsenic biogeochemistry, as well as the development of new bio-technologies for water treatment in rural communities impacted by mining activity.

1. Introduction

Heavy metals in aquatic environments is increasing due to industrialization and unconcerned polluted process. Some metals are classified as toxic to human and living environment. They are of particular concern in the treatment of industrial wastewaters including arsenic (III) and (V), cadmium (III), chromium (III), etc (Srisorrachatr, 2017) Arsenic is a toxic element for humans and other living organisms. By contaminating natural sources of water, it affects their use, the development of sustainable agriculture and the sustainability of the rational use of soils, which is reflected in the lagging socio-economic growth of the affected region (Litter et al., 2009). The toxic effects of As are related to its chemical form and its oxidation state: its organic form is less toxic. On the other hand, in its inorganic form it occurs in two redox states: the reduced form, arsenite (III), and the oxidized form arsenate (V), both of which are toxic to most organisms. Arsenic is commonly found in natural waters in its dissolved form as oxyanions. As (V) is found in its H_3AsO_4 form and its corresponding dissociation products ($H_2AsO^-_4$, $HAsO_4^{2-}$ and AsO_4^{3-}). As (III) appears as H_3AsO_3 and its corresponding dissociation products (H_4AsO^{3+}, H_2AsO^{3-}, $HAsO_3^{2-}$ and AsO_3^{3-}), (Campos and Valenzuela.,

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2007). Within the environment, the most toxic chemical form is arsenite (AsIII) as it is more soluble and more mobile. Arsenate (AsV) is able to adhere to the surface of several minerals, such as ferrihydrite and alumina (Smedley and Kinniburgh., 2002) The mobilization of As in the environment occurs through the combination of natural processes such as weathering, biological activity and volcanic emissions, as well as through a group of anthropogenic activities (Hu and Gao., 2008). It has been reported that microorganisms present in water and soils play a fundamental role in the biogeochemical cycle of As, which influences its behavior in the environment, since they show differences in solubility or bioavailability and toxicity depending on their chemical form (Nriagu., 1994). These microorganisms have developed various mechanisms of tolerance to the toxicity of As. Bacteria metabolize As by oxidation, reduction and methylation, etc. (Smedley and Kinniburgh., 2002). Arsenite oxidase is a heterodimeric enzyme (AioA and AioB), which catalyzes the oxidation of As (III) (Anderson and Williams., 1992). Heterophilic bacteria have been reported in the oxidation of As (III), e.g. Agrobacterium albertimagni and Alcaligenes sp. (Salamassi et al., 2002), while the Rhizobium bacterium generates energy by using As (III) as an electron donor (Santini and. Sly., 2000). Arsenate reductase (ArsC) is a small protein that can catalyze the conversion of As (V) to As (III) (Richey and Chovanec., 2009). Tolerance or transformation mechanisms in some bacteria include a chromosomal or plasmid coded *ars* operon with three or five genes. The operon includes a regulatory gene (ars R), a coding gene for a transmembrane ejection pump specific for arsenite (ars B) and a coding gene for an arsenate reductase (arsC) where the reduction from As (V) to As (III) is carried out by the same (Aditi and Sachin., 2016).

The study area is the mining district Xichú, Guanajuato (La Aurora Mine), which is located 128 km northeast of the capital of the State of Guanajuato. The municipality is adjacent to the states of San Luis Potosí, to the northeast, and Queretaro, to the east, and belongs to the hydrological region number 26 corresponding to the sub-region of the Upper Panuco River, the Upper Panuco Basin, Santa Maria River and the Tamuín River. Records suggest that there has been mining activity in Xichú since the sixteenth century, leaving approximately one million tons of waste around the natural reserve Sierra Gorda of Guanajuato. This waste (mine tailings) is disposed on one side of the Xichú River. Due to its proximity, the local population is exposed to possible contamination from potentially toxic elements (PTE) (Salas, 2014), in this case As. Around the world, it is estimated that more than 40 million people are at risk of drinking water contaminated with As (Nordstrom., 2002).

Normally, it´s use it phisico-chemicals treatments to remove several metallic ions included arsenic from water, for instance, ion exchange, filtration, reverse osmosis, etc; but they have many environmental implications and also are expensive (Denisova et al. 2017) for rural communities.

For these reasons, this study involved the isolation of microbial strains from the Xichú River, their molecular identification and amplification of As-oxidizing or -reducing genes, as well as the contribution to developing bio-treatments to remove arsenic from water in rural communities where mining activity is principal source of contamination.

2. Materials and Methods

2.1 Description of the site and Sampling

Samples were collected from the Xichú River located within the municipality of Xichú, Gto, (100°03 of Longitude West of the Greenwich Meridian and at 21°18¨, Mexico). Eleven microbiological samples were obtained, four water samples: AJ (14N 392 952 E2358733N), J (14N 392914E 2359028N), DJ (14N 393054E 2359191N) and OA (14N 402757E 2371745N), four sediment samples (AJS, SM, EJS and JS) and one biofilm (BiofJ). For water monitoring sampling the Mexicans normativity NMX-AA-14-1980 and NMX-AA-132-SCFI-2006 were taken into consideration. The plastic containers used were first treated with HNO_3 10% for 24 hours, before being washed with distilled water. For the collection of water samples, the plastic container was introduced into the water body until it was filled and sealed before being removed from the water body. In the case of sediment samples, a previously sterilized metallic spatula was used, and samples were deposited in sterile 50 ml falcon tubes. Finally, biofilm samples (Van Hullebusch et al., 2003) were collected in the same way as sediment samples.

2.2 Physico-chemical characterization of Water Samples

The quantification of total arsenic was carried out in two stages: the first one was in-situ with an Arsenator Wagtech Arsenator® (WHO, 10 ppb μl^{-1}), besides the quantification of S^{2-}, SO^{4-}, Fe^{2+}, pH, temperature (°C), and dissolved oxygen (O.D), with a HACH DR 3900 and a Thermo scientific potentiometer (Water analysis) respectively. The second stage was in the laboratory, where a Perkin Elmer atomic absorption equipment model PinnAcle 900T with hydride generator was used for the quantification of total arsenic. Nitrites and other heavy metal cations such as Zn^{2+}, Mn^{2+}, Pb^{2+}, Cd^{2+} and NO_2^- were also quantified.

2.3 Isolation of Microorganisms

Nutritional Agar (AN) was prepared with different concentrations of Arsenate (Sodium arsenate, Na_3AsO_4) and Arsenite (Sodium arsenite, $NaAsO_2$) which ranged from 3.5 mM to 20 mM. Incubation conditions for all samples were 37°C for both aerobic and anaerobic samples. In the case of the strains exposed in anaerobiosis, an anaerobic chamber was used with GasPack. For the first inoculation they were taken directly from the microbiological water sample (50, 100 and 200 µl). In the case of sediments and biofilms, a sample was taken and resuspended in 20 ml of sterile water, from which 50 and 100 µl was later taken for inoculation.

2.4 Reduction-Oxidation of As by Microbial Action

In order to determine the oxidation or reduction of arsenic various colorimetric tests can be applied in order to infer, according to the colouring presented, the type of mechanism used by each of the bacterial isolates to resist the toxicity of the metalloid, either by means of the reduction of As (V) through the reaction with $KMnO_4$ to As (III) (Salmassi et al., 2002). Where the first steps is the preparation of a fresh culture 24 hours before the assay, followed by a preparation of a medium with low concentrations of As(III) and As(V), 1 -3 mM each medium for a subsequent inoculation. Incubation conditions were 72 hours, 37°C and 120 rpm. Ones obtained the cultures it were centrifuges 10,000 rpm during 5 minutes, it's necessary to take out 3 ml of the supernatant followed by an addition of $KMnO_4$ (10mM).

2.5 Growth Kinetics

In order to observe the growth time of the isolates, 4 growth curves will be used, as described below:
Growth kinetics in Nutrient Broth (NB): in 50 ml falcon tubes add 30 ml of NB medium and incubate at 37°C at 120 rpm for 7 days. Each assay it were measured every 24 hours.
Growth kinetics in YEM with As(III) 10 mM. Incubation conditions: in 50 ml falcon tubes add 30 ml YEM medium added with 10 mM Sodium arsenite and incubate at 37°C at 120 rpm for 8 days.
Growth kinetics in YEM with As(V) 10 mM. Incubation conditions: In 50 ml falcon tubes add 30 ml of YEM medium added with 10 mM Sodium Arsenate and incubate at 37°C at 120 rpm for 8 days.
Growth kinetics in YEM with As(III) and (V) 10 mM. Incubation conditions: in 50 ml falcon tubes add 30 ml of added YEM medium with a concentration of 10 mM of both As(III) and As(V) and incubate at 37°C at 120 rpm for 8 days.

2.6 Molecular identification of Microorganisms and Amplification of ars and aox Genes

The extraction of the genomic DNA of the bacteria was carried out based on the protocol described by Murray and Thompson (1980), where the technique of extraction with Cetyl Trimethyl Ammonium Bromide (CTAB)/NaCl was used. Once the extraction was finished, an electrophoresis was performed in a 1% agarose gel with ethidium bromide to check the quality and concentration of the DNAg. This was followed by the amplification of the Ribosomal 16S gene by PCR,and the purification of the PCR product using the GE Healthcare kit, illustrated, GFX PCR DNA and Gel Band Purification, in which protocols and supplier suggestions are followed. The purified PCR products were sent to the National Laboratory of Agricultural, Medical and Environmental Biotechnology (LANBAMA) in San Luis Potosí, which is operated and supported jointly by the Division of Molecular Biology and the Division of Environmental Sciences of the Instituto Potosino de Investigación Científica y Tecnológica (IPICYT). Finally, the data was corroborated with Gen Bank.The amplification of the ars and aox genes was also carried out using the following methodology, which was used on each of the isolated bacterial strains in order to detect the presence or absence of those genes that are directly related to the resistance to arsenic (As). In order to do this, PCR was mixed with Water miliQ, Buffer PCR (10X), MgCl2, dNTPs, the initiators ars A Forward and ars Reverse, in the case of arsenate reductase (arr A), and the initiators aox A and aox B in the case of arsenite oxidase, DNAg and Taq Polymerase (Invitrogen). The reaction was carried out in a Thermocycler (LabNet), at an alignment temperature of 60 °C, ending with the Extension Phase at 72 ºC for 1 minute. Finally, a cycle was applied for a final extension of 10 min. at 72ºC and finally a maintenance stage was programmed at 4°C.

3. Results

3.1 Physico-chemical characterization of Water Samples.

The results of the in-situ water characterization are shown below in Table (1).

Table 1. In-situ physicochemical characterization of water samples in low season (April) and rainy season (September). Ast was measured in laboratory for AAS.

Parameters	Samples - April					Samples - September					
	AJ	J	EJ	DJ	OA	AJ	J	EJ	DJ	MZ	RT
SO_4^{2-}mg L^{-1}	110	132.5	145	160	122.5	12.3	17.6	12	15.3	24	18
pH	9.3	9.55	9.5	10.2	7.5	8.43	8.38	8.34	8.66	8.6	8.77
S^{2-}mg L^{-1}	0	0	0	0	0	0.09	0.15	0.14	0.1	0.14	0.07
C.E µS cm^{-1}	630	957	653	661	647	155.9	156.6	157.3	161.4	188.6	230.8
O.D mg L^{-1}	5.7	4.68	4.18	4.45	4.2	2.08	1.67	0.8	1.6	1.2	0.96
T(°C)	20	20.5	22.6	17.8	27.1	17.8	17.7	17.7	18.1	21.1	22.9
Ast µg L^{-1}	35	40	50	98	8.9	1	0	0	0	0	0
Fe^{2+}mg L^{-1}	0.04	0.036	0.042	0.08	0.022	0.34	0.39	0.73	0.34	0.56	0.24
NO_3^-mg L^{-1}	9.6	1.25	6.8	0.9	4	2.6	2.9	2.1	4.4	5.8	5.3

3.2 Isolation of Microorganisms

From the collected samples, a total of 16 strains were isolated: 10 strains from water samples, 5 strains from sediments and 1 strain from biofilms. Most of them resisted up to 20 mM in solid media and 10 mM in liquid media of arsenite and arsenate except for one strain from the OA water sample, which is strictly anaerobic and only resisted up to 10 mM from both As species. In these study only 2 strains were analyzed.

3.3 Oxide- Reduction by Microbial Action

In relation with colorimetric test, only one strain was observed from biofilm sample capable of reducing As (V), B13. (Figure 1), The reaction with $KMnO_4$ turns the medium pink in the presence of As (V), whilst it turns a light yellow if the reduction to As (III) occurs. (Salmassi et al., 2002).

Figure 1. Colorimetric test with $KMnO_4$ for the observation of reducing strains of As (V).

3.4 Growth Kinetics

The growth kinetics of the different behaviors of the isolates are presented below. Figure 2 corresponds to strain A1, grown in YEM without As and with As (V), 10 mM; As (III), 10 mM and the mixture of the two salts of As at a final concentration between them of 10 mM. The growth kinetics of this A1 strain in all growing conditions was similar, without it being affected by any of the arsenic salt..

Figures 3, corresponding to strain B13 grown in YEM without As and with As (V), 10mM; As (III), 10 mM and the mixture of the two salts of As to a final concentration between both of 10 mM. The growth kinetics of this strain show that it grows better according to this order (where the symbols represent, = equal kinetics and > better growth): As (V) > As (III) = mixture > Control.

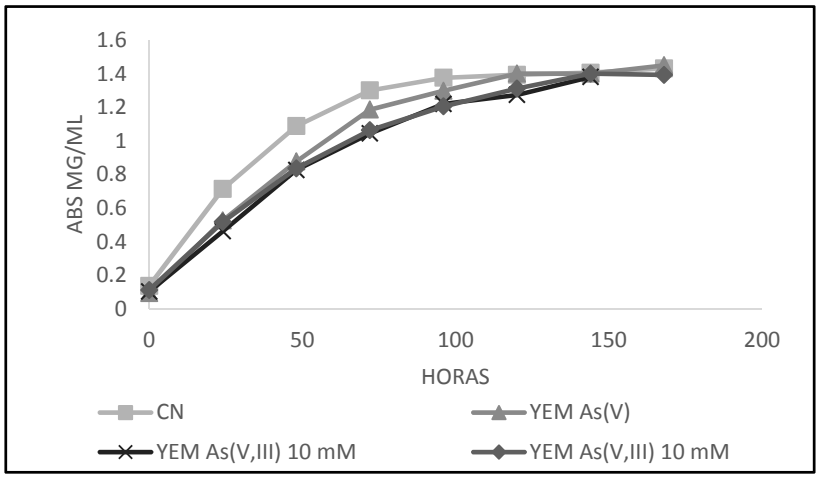

Figure 2. Growth kinetics of strain A1, in NB medium without As and YEM medium with As.

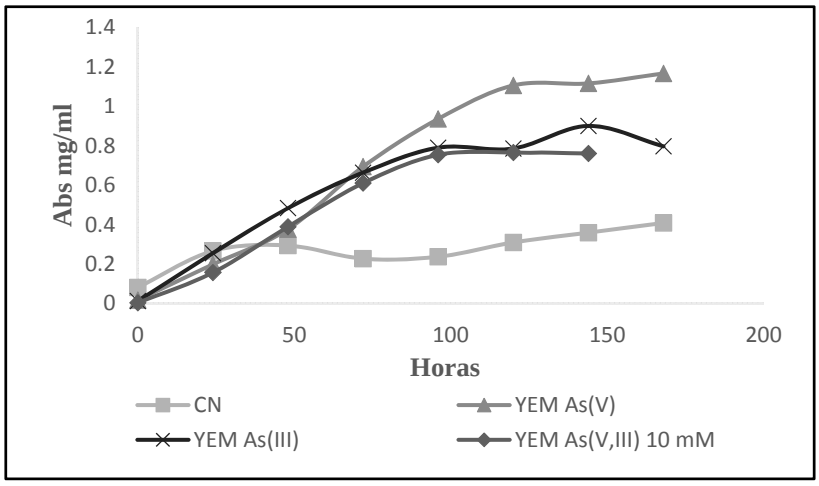

Figure 3. Growth kinetics of strain B13, in NB medium without As and YEM medium with As.

3.5 Molecular identification of Microorganisms.

The molecular identifications of 6 isolated strains are presented, the results are shown in Table (2).

Table 2. Identification of isolated strains, resistance to As and the presents of genes ars and aox.

Key	Identity	Accession Number	Genus and Species	Genes Presence	
				ars	aox
A1	99%	NR_025730.1	Rhodococcus gordoniae	-	-
B13	99%	NR_042263.1	Microbacterium hydrocarbonoxydans	-	-

4. Conclusions

In this study it has been possible to report bacterial species resistant to As: *M*icrobacterium hydrocarbonoxydans and Rhodococcus gordoniae, ich one resist until 20mM of arsenic salts As(III) and As(V) respectively.The colorimetric test with $KMnO_4$ it was positive for these strains. Nevertheless, the ars and aox genes were not detected through PCR test in these microorganisms. Therefore, it can be deduced that their expression is not a factor that favors their arsenic resistant or their proliferation in environments contaminated with this metalloid.

Likewise in the strain R. gordoniae, isolated in this investigation, neither it detected any of these genes mentioned,(ars and aox).This leads us to the hypothesis that the strain R. gordoniae could bio-absorb on the surface of the cell the species of As (V) and (III) or use it as an energy production. According with the grown kinetic of both microorganisms they are greatest arsenite oxidizer. That's means that they are great candidates for remove arsenic from water. The relevance of the isolation of microorganisms from contaminated sites, in the case of this study in the municipality of Xichú, Gto, is their potential use to remove arsenic from water in this area with low economic resources. It has been proven that, these biotechnologies could have a lower cost than chemical and physical treatments, and also it could be implemented in marginalized sites, they are sustainable and therefore without environmental implications.It's extremely important identify the capacities of each strain for arsenic transformation, if it want to use a biological treatment for arsenic removal.According with the concentration of As that it's found during the dry season (98 μg L^{-1}), this zone around the mine "La Aurora" is highly toxic due to the presence of heavy metals and metalloids in its surface waters, therefore, it can affect to the rural communities that live around the mine.

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