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Isolation and Selection of Sulfur-oxidizing Bacteria for the Treatment of Sulfur-containing Hazardous Wastes

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A total of 75 microorganisms were obtained from high-sulfur content environmental samples using different sulfur sources. Fifty-four of them had the ability to oxidize sulfur at 1% (w/v) in liquid culture, however only three of them AZCT-M125-5, AZCT--M125-6, and AZCT-M125-7 were able to grow autotrophycally using elemental sulfur at concentrations higher than 1 % and up to 9 % (w/v) as energy source. They produce more than 300 mg sulfate/L. Also, these microbial cultures were able to produce sulfate within pH 3 to 7. Analysis based on 16S rRNA gene sequences indicated that microbial cultures AZCT-M125-5 and AZCT-M125-6 were closely related to *Acidithiobacillus thiooxidans* while identification of AZCT-M125-7 was not possible. According to the results, these three microorganisms can be excellent candidates for the future development of alternative biotechnological processes for the treatment of hazardous wastes containing sulfur.

Key words:
Sulfur-oxidizing, isolates, chemolithotrophic, acidophilic

Introduction

Sulfur compounds are among the major pollutants in the environment since they cause adverse impacts on ecosystems. As widely known, the emission of hydrogen sulfide (H_2S) and reduced sulfur compounds from anthropogenic sources produces corrosion to metallic facilities, bad odor and, under specific conditions, they are toxic to human health.[1,2] According to Mexican environmental regulations (NOM-148-SEMARNAT-2006: Air pollution. Recovery of sulfur from the oil refining process) all gas streams containing sulfur compounds must be treated.[3,4] To reduce the damage caused by sulfur contaminants, several physicochemical processes are being employed which, in most cases, are expensive and are not environmentally friendly.[5,6] Biological techniques have appeared as potential alternatives to solve this problem since there are microorganisms able to transform and/or capture sulfur compounds, such as chemolithoautotrophic sulfur-oxidizing microorganisms (SOM). Chemolithoautotrophic sulfur oxidizing-microorganisms use carbon dioxide as carbon source and obtain their energy from the oxidation of reduced sulfur compounds such as hydrogen sulfide, sulfite, sulfur, thiosulfate, and several polythionates.[7,8]

The sulfur-oxidizing prokaryotes are phylogenetically diverse. In the domain *Archaea*, aerobic sulfur oxidation is restricted to members of *Sulfolobales*, and, in the domain *Bacteria,* sulfur is oxidized by aerobic lithotrophs or anaerobic phototrophs.[9] For example members of the genus *Bacillus, Beggiatoa, Thiothrix, Thermothix, Thiovolum, Acidianus, Sulfolobus, Thioalcalimicrobium, Thioalkallividrio,*[10,11,12] and *Thiobacillus* can be classified as *Acidithiobacillus, Thermithiobacillus,* and *Halothiobacillus.*[13] Sulfur-oxidizing fungi have usually been considered as chemoorganotrophs that utilize organic compounds as the major carbon and

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energy source. However, the ability to use inorganic sulfur compounds has been reported in various groups of fungi.[14] Sulfur-oxidizing microorganisms have a wide metabolic diversity and have been isolated from hypersaline, as well as from acid, neutrophilic, and alkalophilic, environments. According to their response to pH, sulfur- oxidizing species include acidophiles (optimum pH 2–5), neutrophiles (optimum pH 6–8), and alkaliphilic (optimum pH 10–11) microorganisms. In addition, they can be found in a wide range of temperatures such as thermophilic (optimal temperature 50–75 °C), psychrophilic (optimal –5 a 5 °C) or mesophilic (optimal temperature 25 to 40 °C) microorganisms.[15] Acidophiles have received much attention because of their important role in acidic metal leaching processes.

The aim of this study was to obtain and select sulfur-oxidizing microorganisms from high-sulfur content environmental samples to propose them as candidates in the research and possible development of biotechnological processes, such as metal leaching from complex and sulfur raw materials or in bio-hydrometallurgical approaches to recover valuable metals/sulfur from industrial wastes. As part of this study, it was also important to evaluate their ability to grow at different sulfur concentrations and pH.

Materials and methods

Culture media and microorganisms

Strains *Acidithiobacillus ferrooxidans* ATCC 53987, *Acidithiobacillus thiooxidans* ATCC 55020, and *Acidithiobacillus thiooxidans* ATCC 8085 were used as reference strains due to their ability to oxidize sulfur.

Different media were used for enrichment and isolation of sulfur-oxidizing microorganisms: ATCC 125 medium consisted (g L^{-1}) of: $(NH_4)_2SO_4$ (2.0), $MgSO_4 7H_2O$ (0.5), $CaCl_2$ (0.25), KH_2PO_4 (3.0), $FeSO_4$ (0.005), and sulfur (10.0); pH was adjusted to 3 with sulfuric acid. Starkey medium was composed of the following salts (g L^{-1}): $(NH_4)_2SO_4$ (3.0), Na_2SO_4 $10H_2O$ (3.2), KCl (0.1), KH_2PO_4 (0.05), $MgSO_4 7H_2O$ (0.5), $Ca(NO_3)_2$ (0.01). This medium includes trace elements (mg L^{-1}): $FeCl_3 6H_20$ (11.0), $CuSO_4 5H_2O$ (0.5), HBO_3 (2.0), $MnSO_4$ H_2O (2.0), $Na_2MoO_4 2H_2O$ (0.8), $CoCl_2 6H_2O$ (0.6), $ZnSO_4 7H_2O$ (0.9);[16] 10 g of elemental sulfur/L was added as energy source. The basal medium was adjusted to pH 2.5 with sulfuric acid.[17] Modified Starkey medium was composed of (g L^{-1}): KH_2PO_4 (3.0), $(NH_4)_2SO_4$ (0.2), $MgSO_4 7H_2O$ (0.5), $CaCl_2 2H_2O$ (0.3), and $FeSO_4 7H_2O$ (0.1). This medium was supplemented with 30 ppb of molybdenum.[18] and 10 g of elemental sulfur/L. The basal medium was adjusted to pH 3 with sulfuric acid;[7] 5 g of sodium thiosulfate/L, instead of elemental sulfur, was added to the Starkey thiosulfate medium. To obtain heterotrophic sulfur-oxidizing microorganisms, 5 g dextrose/L was added to the Starkey medium.[19] Thiosulfate mineral medium was composed of (g L^{-1}): NH_4Cl (0.1), KH_2PO_4 (0.05), $MgSO_4 7H_2O$ (0.02), $NaS_2O_3 5H_2O$ (0.4), yeast extract (1.0), the pH was adjusted to 3 with sulfuric acid.[20] The 9K medium was composed of (g L^{-1}): KH_2PO_4 (0.4), $CaCl_2 2H_2O$ (0.2), $MgSO_4 7H_2O$ (0.4), $(NH_4)_2SO_4$ (0.4), $FeSO_4 7H_2O$ (33.3). The basal medium was adjusted to pH 1.5–2 with sulfuric acid.[21,22] Solid medium was prepared by adding agar-agar at 18 g L^{-1}, except the 9K medium for thermophiles.

Sample collection

A total of 8 samples, both liquid and solid, were collected from soil, water, and industrial wastes (i.e. catalysts) with high sulfur-content from different regions of Mexico. Samples were collected in sterile plastic vials, stored at room temperature, and transported to the laboratory. Solid samples were crushed and ground in a mortar to reduce particle size and then homogenized prior to their use. Liquid samples were used directly without treatment. Physical and chemical analyses of samples revealed a pH range from 2.6 to 5.3, electric conductivity from 1.13 to 14.25 dS m^{-1}. Metal analyses showed the presence (in mg L^{-1}) of: Al (4 – 9730), As (12 – 77), Ca (43 – 2246), Cr (4 – 153), Cu (not-detected – 70.5), Fe (1.8 – 4493.5), K (9.2 – 5552.7), Mg (14 – 386), Na (12 – 585), Ni (not-detected – 43.75), P (6 – 169), V (0.1 – 10), Zn (5 – 672).

Isolation and morphology observation

For the isolation of sulfur-oxidizing microorganisms, the culture was enriched using 5 g of solid or 5 mL of liquid samples, placed in 125-mL Erlenmeyer flasks containing 30 mL of Starkey medium, Starkey thiosulfate medium, thiosulfate medium, ATCC 125 medium, 9K medium, or modified Starkey medium. Inoculated flasks were incubated at 30 °C and 140 rpm for 7 days or until turbidity development, which was related to microbial growth. Afterwards, decimal dilutions were prepared from 10^{-2} to 10^{-4} and each dilution (aliquot of 0.1 mL) was spread on plates containing Starkey--sulfur solid medium, Starkey thiosulfate solid medium, mineral thiosulfate solid medium, or ATCC 125 solid medium.

Petri dishes were incubated at 30 °C until observing microbial growth.[17] The isolated colonies

were selected based on different colonial morphology and were plated in fresh medium to obtain pure cultures. Polarized light microscopy (Axio Scope.A1) was used to determine purity of the colonies by means of Gram staining and microculture for filamentous fungi. Based on isolation and microbial growth results (see Table 1), modified Starkey medium was selected for further studies.

Table 1 – *Environmental samples codification, culture media and isolates*

Environmental Sample (coded)[a]	Culture media[b]	Number of isolates
IWD, IWW, FS, TS, WW	Starkey media, pH 2.5	4
IWD, IWW, FS, TS, WW	Modified Starkey media, pH 3	26
IWD, IWW, FS, TS, WW	Starkey media-dextrose, pH 3	7
IWD, IWW, FS, TS, WW	Starkey thiosulfate media, pH 3	4
IWD, IWW, FS, TS, WW	thiosulfate mineral media, pH 3	6
TC, CU, GL	9K, pH 2	14
TC, CU, GL	ATCC 125, pH 3	14

[a]Abbreviations: IWW, industrial waste, wet; IWD, industrial waste, dry; TS, Tlaxcala soil; FS, forest soil; WW, wastewater; TC, Tourist Camp at National Park "the sulfur"-; CU, Curritaco at National Park "the sulfur"; GL, Green Lagoon at National Park "the sulfur".

[b]See culture media composition

Evaluation of sulfur-oxidizing activity by isolates

Inocula of 54 microorganisms (21 isolates were eliminated because of poor microbial growth, see results) and three reference strains were prepared in 50-mL antibiotic-type bottles containing 10 mL of modified Starkey mineral medium, the inoculated flasks were left for 6 days to achieve appropriate growth, then an inoculum of 3 mL was transferred to fresh medium to evaluate their sulfur-oxidizing ability in batch culture, using 125-mL Erlenmeyer flasks containing 30 mL of modified Starkey medium (elemental sulfur at 1 %, w/v). Fungi were grown in modified Starkey media supplemented with dextrose at 0.5 %. Flasks were incubated at 30 °C and 140 rpm for 7 days. After this period, sulfur oxidation was evaluated, determining sulfate (SO_4^{2-}) concentration in the supernatant according to the NMX-k-436-1977 method.[23] The production of sulfuric acid was also evaluated in the supernatant by pH measurement using a digital potentiometer (Thermo Scientific, Orion). In this phase of experimentation and due to the high amount of isolates obtained, only those microorganisms able to grow faster and that presented the higher sulfur-oxidizing activity were selected for more studies.

Effect of sulfur concentration and pH with selected isolates

Two different experimental sets were separately prepared to determine the maximum sulfur concentration and pH range at which selected isolates present an important sulfur-oxidizing activity. Seven microorganisms previously selected, were used to evaluate the effect of sulfur concentrations (AZLV-M125-1, AZLV-M125-2, AZCT-M125-5, AZCT-M125-6, AZCT-M125-7, AZCT-M125-9, and AZCU-M125-13) and three reference strains (*Acidithiobacillus ferrooxidans* ATCC 53987, *Acidithiobacillus thiooxidans* ATCC 55020, and *Acidithiobacillus thiooxidans* ATCC 8085). The first set was prepared in 125-mL Erlenmeyer flasks containing 30 mL of modified Starkey medium (pH 3), and different elemental sulfur concentrations (1, 3, 4, 5, 7, and 9 % w/v). An inoculum of 10 % of each one of the selected microorganisms and the reference strain was used. Erlenmeyer flasks were incubated at 30 °C and 140 rpm for 7 days. Control flasks included modified Starkey media containing different sulfur concentrations but no inoculum was added. After the incubation period, the microbial oxidation of elemental sulfur was evaluated by the production of sulfate (SO_4^{2-}) and pH decrease in the supernatant as mentioned above.

In the case of the evaluation of pH effect on sulfur-oxidizing activity only AZCT-M125-5, AZCT--M125-6, AZCT-M125-7 were evaluated which were prepared similarly as was mentioned previously but initial pH of modified Starkey medium was adjusted to pH values of 3, 4, 5, 6, and 7. Control flasks included modified Starkey media adjusted to different pH but no inoculum was supplemented. After the incubation period, the microbial oxidation of elemental sulfur was evaluated by the production of sulfate (SO_4^{2-}).

Amplification and sequencing of 16S rRNA gen

Biomass of 50 mL of the strains AZCT-M125-5, AZCT-M125-6, and AZCT-M125-7 was harvested by centrifugation at 4000 rpm for 5 min., the cellular package was washed with 1 mL of sterile and deionized water and centrifuged at 4000 rpm for 5 min. Cell lysis was performed by chemical breakdown using 400 μL of a 10 mM of Tris-HCl pH 8.0, 1 mM EDTA, 10 mM NaCl, SDS at 1 % solution and mechanical shaking with glass beads (0.5 g). Later, the mixture was vortexed at 8 pulses of 30 sec. and left to rest in ice for periods of 30 s. Then 400 μL of a phenol-chloroform-isoamyl

alcohol solution at a ratio of 25:24:1 and 200 μL of TE buffer were added and vortexed, the resulting mixture was centrifuged at 14000 rpm for 5 min. The supernatant was recovered in a microtube, to which 1 mL of absolute ethanol was added and mixed by inversion. The above mixture was then cooled for 20 min. at –20 °C, and centrifuged at 14000 rpm for 5 min. The ethanol was removed and the package washed with 1 mL of ethanol at 70 %. The DNA sample was dried in a laminar flow hood at room temperature. DNA sample was suspended in 50 μL of sterile and deionized water.

The presence and quality of DNA was determined by chromatographic separation on agarose gel at 1 %.[24] By means of PCR, a 1540 bp (base pairs) fragment was amplified, corresponding to the 16S rRNA of the consortium members under study, using universal primers 8 and 1492 previously recommended.[25] A thermal cycler TC-3000 (Techne, Applied Biosystems, USA) was used. Every 50 μL contained 2 μL of dNTP (10 mM), 5 μL 10X buffer solution, 6 μL $MgCl_2$ (50 mM), 2 μL of each primer (10 pM), 2 μL of DNA sample (50-100 ng), 1 μL Taq polymerase (5 U), and sufficient purified water to complete the final volume. DNA amplification was carried out under the following conditions, initial denaturation at 94 °C for 5 min.; 30 cycles of 92 °C for 1 min., 55 °C for 1 min., and 72 °C for 1 min.; and a final elongation cycle at 72 °C for 5 min. 16S rRNA of all samples was purified using a commercial reagent kit QIAquick gel extraction kit (Qiagen N. V., Germany). Sequencing of the purified PCR products was performed with an automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems, California, USA) at the Instituto de Neurobiologia UNAM, Querétaro, México.

The partial sequences were then subjected to a BLAST search version 2.2.3.[26] to search for the taxonomic hierarchy of the sequences. A collection of taxonomically related sequences obtained from the National Center for Biotechnology Information (NCBI) Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) was used to perform a multiple alignment analysis with CLUSTAL X.[27] Multiple alignments were manually edited using SEAVIEW software.[28] Only common 16S rRNA gene regions were included in the phylogenetic tree and similarity analysis using the Kimura 2-parameter model was performed with the MEGA version 5.05 software.[29] The phylogenetic tree was constructed using the UPGMA method and 1,000 bootstrap replications were assessed to support internal branches. The similarity percentages among sequences were calculated using BioEdit 7.0.5.2 software.[30] The genus and species limits were 95 and 97.5 %, respectively, according to the suggested criteria.[31]

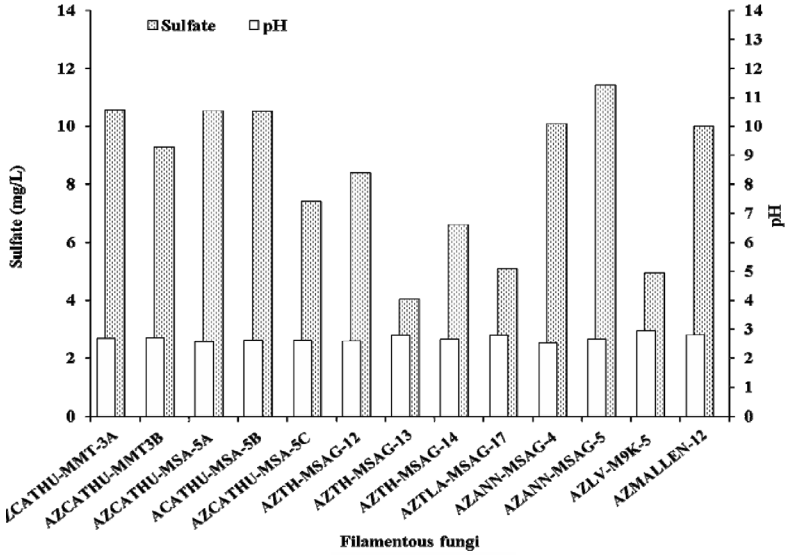
Results and discussion

Isolation and morphology of microorganisms

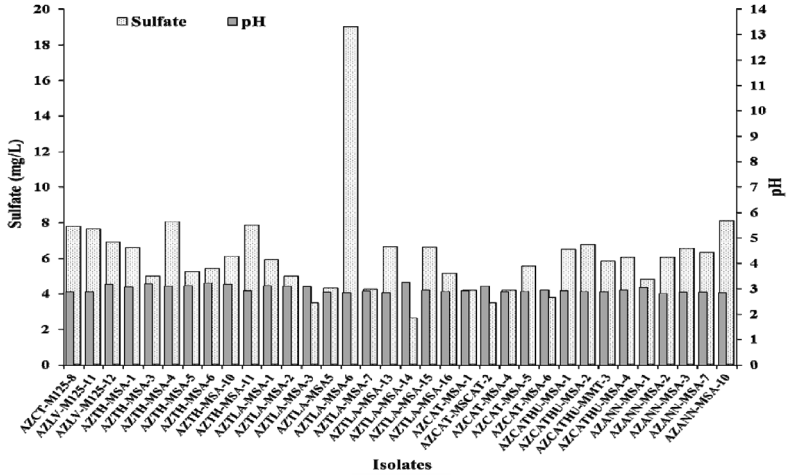
Table 1 shows the results of isolation from 8 different samples and different culture media. As can be observed, the use of different media allow to obtain a total of 75 isolates; microbial diversity was composed of bacteria, actinomycetes, yeasts, and filamentous fungi (hyaline and demateaceus). The predominant microbial group was Gram-negative, short rod-shaped and small cocci to short rods as single or pair cells and some Gram-positive non-spore forming, and spore forming long-rod-shaped bacteria, and finally actinomycetes with and without mycelia fragmentation. A higher amount of isolates was obtained with modified Starkey media; hence this media was selected for later studies. Reports in the literature mention species belonging to the genera *Thiobacillus, Thiomicrospira; Thiosphaera*, and other species such as *Paracoccus, Xanthobacter, Alcaligenes*, and *Pseudomonas* that have a chemolithotrophic growth in the presence of inorganic sulfur,[12,32] also species related to the genera *Acidithiobacillus*, *Sulfobacillus, Bacillus,* and Archaea such as *Ferroplasma,*[10,11,33] and actinomycetes such as *Streptomyces,*[34] have been reported. In the case of sulfur-oxidizing eukaryotic organisms, most isolates corresponded to yeasts with variable size and shape. Some genera of yeasts such as *Ebaryomyces, Saccharomyces,* and *Rhodotorula* have been reported as sulfur-oxidizing microorganisms.[14] In the present study, from the 13 sulfur-oxidizing fungi, 12 were identified as follows *Aspergillus sp, Aspergillus niger, Penicillium sp* (4 fungi)*, Geotrichum sp, Rhizopus sp, Paecilomyces variotii, Cladophialophora, Mucor sp* and *Alternaria sp.* Only one fungus has not been identified. The ability of conversion of inorganic sulfur compounds has been reported in various groups of fungi, for example *Alternaria, Aureobasidium, Penicillium, Fusarium, Absidia,* and *Zygorhynchus.*[14]

Evaluation of sulfur-oxidizing activity by isolates

As it was mentioned previously, 75 microorganisms were isolated from 8 environmental samples using different culture media but only 54 were evaluated for their ability to oxidize elemental sulfur to sulfate, that is, 21 isolates that came from media 9K were eliminated because of their poor growth after 7 days of incubation. Among the 54 isolates assayed, the thirteen filamentous fungi



F i g . 1 – *Production of sulfate and final pH by filamentous fungi after 7 days of incubation at 30°C, 140 rpm in modified Starkey media and dextrose at 0.5 %*



F i g . 2 – *Production of sulfate and final pH by bacterial isolates after 7 days of incubation at 30°C, 140 rpm in modified Starkey medium*

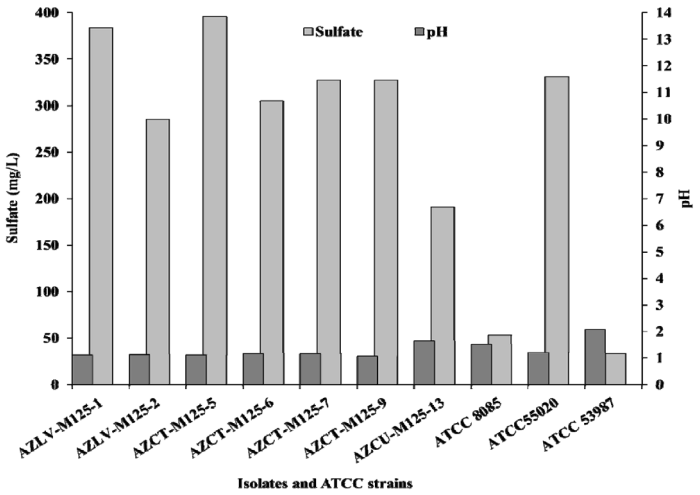
were able to produce sulfate in the order of 4 to 11 mg L^{-1} (Figure 1). Lawrence and Germida[35] proposed that heterotrophic sulfur oxidizers may be categorized into at least three major groups: (i) those that oxidize S° producing thiosulfate as their predominant end product, (ii) those capable of oxidizing S° producing sulfate, and (iii) those capable of oxidizing thiosulfate to sulfate; suggesting that a mixed population of heterotrophs probably plays the dominant role in sulfur oxidation in many aerobic agricultural soils.[35] Regarding to final pH, it can be observed (Figure 1) that for fungi cultures pH values were within 2.5 – 2.8, that is, the pH was not significantly affected due to sulfur-oxidizing activity, also an important finding was the fact that no correlation was found between sulfate production and pH decrease.

For bacteria, all of them (41) grew autotrophically using elemental sulfur as energy source (Figure 2 and 3); Figure 2 shows that 34 isolates presented levels of sulfate production in the order of 3 a 19 mg L^{-1}, while in Figure 3 is showed that 7 isolates have a sulfate production within 190 to 380 mg L^{-1}, that is, only 7 isolates were able to oxidize sulfur to sulfate at higher extent. Sulfate production of reference strains *Acidithiobacillus thiooxidans* ATCC 8085 and *Acidithiobacillus ferrooxidans* ATCC 53987 was lower than 53 mg L^{-1} and only *Acidithiobacillus thiooxidans* ATCC 55020 has a similar sulfate production than isolates showed in Figure 3, which corresponded to 330 mg L^{-1}. It is important to mention that this experimentation was done using 1 % of sulfur.

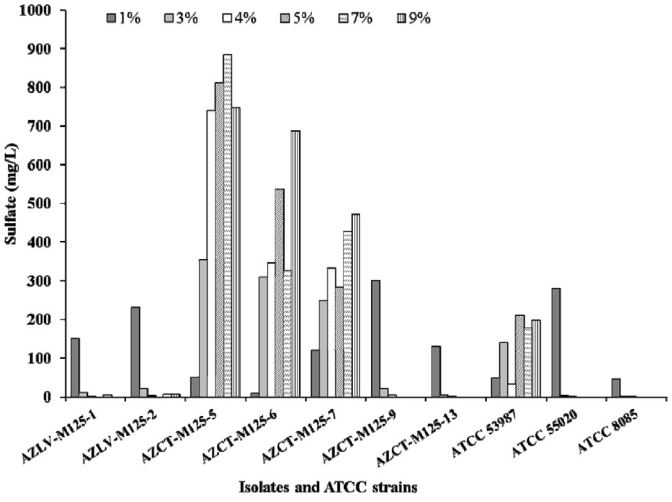
As mentioned above, the initial pH for modified Starkey media was 3; isolates which produce lower amounts of sulfate (Figure 2) decrease the final pH to values within 2.9–2.7 while those isolates which were able to produce higher amounts of sulfate (Figure 3) decrease the final pH values to 1.1 – 1.6, however any direct correlation was observed between sulfate production and pH decrease. For reference strains, final pH values were within 1.2 – 2.0 and equally no correlation was found between sulfate production and decrease in pH. In view of this information and taking into consideration sulfur-oxidizing activity (sulfate produced) of the 54 microorganisms assayed; only 7 isolates and reference strains were later evaluated.

Effect of different sulfur concentrations

Seven isolates and three reference strains were evaluated for their sulfur-oxidizing activity at different sulfur concentrations: AZLV-M125-1, AZLV--M125-2, AZCT-M125-5, AZCT-M125-6, AZCT--M125-7, AZCT-M125-9, AZCU-M125-13, *Acidithiobacillus ferrooxidans* ATCC 53987, *Acidithiobacillus thiooxidans* ATCC 55020 and *Acidithiobacillus thiooxidans* ATCC 8085. It can be seen in Figure 4, that only three isolates AZCT-M125-5, AZCT-M125-6, AZCT-M125-7 and one reference strain *Acidithiobacillus ferrooxidans* ATCC 53987



F i g . 3 – *Production of sulfate and final pH by bacterial isolates after 7 days of incubation at 30°C, 140 rpm in modified Starkey medium*

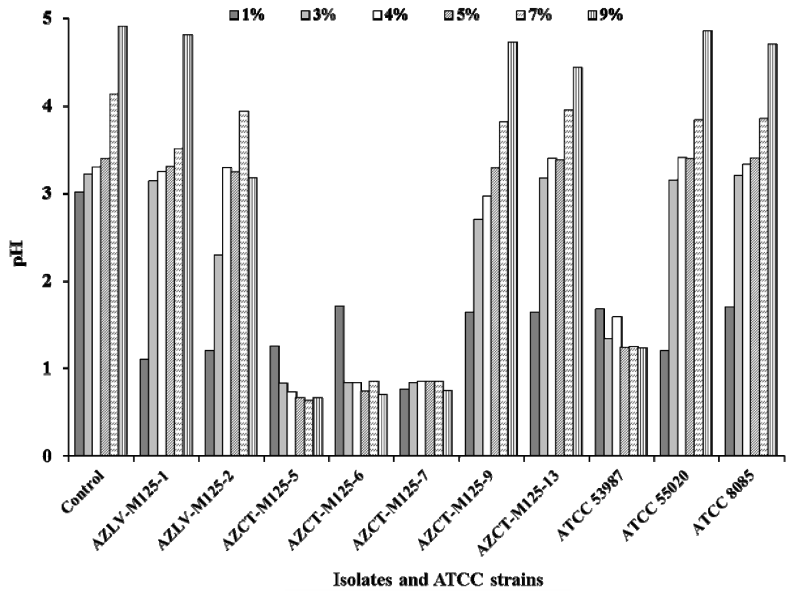


F i g . 4 – *Sulfur oxidation and pH evaluation by sulfur-oxidizing isolates at different sulfur concentrations after 7 days of incubation at 30°C, 140 rpm in modified Starkey medium*

show that ability to oxidize sulfur at different concentrations (1 to 9 %). Most of all microorganisms were able to produce sulfate at 1 % but when sulfur concentration increases to 3 % sulfate production falls except for the above mentioned microbial cultures. Figure 4 also reveals that isolate AZCT--M125-5 was able to produce the highest amount of sulfate 885 mg L^{-1} at a sulfur concentration of 7 % (w/v) in comparison with 327 and 426 mg L^{-1} for isolates AZCT-M125-6 y AZCT-M125-7 at the same concentration and incubation conditions. However at the highest sulfur concentration evaluated (9 %), the sulfate production for isolates AZCT-M125-5, AZCT-M125-6, and AZCT-M125-7 corresponded to 747, 687 y 470 mg L^{-1} respectively. The reference strain, *Acidithiobacillus ferrooxidans* ATCC 53987, showed a sulfate production of 177 and 196 mg L^{-1} at a sulfur concentration of 7 and 9 %, respectively. The isolate AZCT--M125-5 showed the highest levels of sulfur transformation or sulfur oxidation, which corresponded to 2.5 to 3 g at concentrations within 4 to 9 %, that is, a sulfur transformation of 60 %. The studied isolates AZCT-M125-5, AZCT-M125-6, and AZCT--M125-7 were able to produce between two and five times (depending sulfur concentration) more sulfate than *Acidithiobacillus ferrooxidans* ATCC 53987 at concentrations of 7 and 9 %.

It is noteworthy to observe that the isolates have a different behavior respect to sulfur concentration, AZCT-M125-5 decrease its sulfur-oxidizing activity when sulfur concentration exceeds 7 % while AZCT-M125-5 and AZCT-M125-6 have a different behavior, for AZCT-M125-6 and AZCT--M125-7 higher sulfur concentration (from 7 to 9 %) improves their sulfur oxidizing ability, although this increase is more evident for AZCT-M125-6. At higher sulfur concentration than 9 % a higher sulfur-oxidizing activity could be expected for AZCT--M125-6 and maybe for AZCT-M125-7 however, higher sulfur concentrations were not evaluated since the possible application involves an industrial waste whose maximum sulfur content is about or less than 8 %. Additional studies could be done to cover a wide range of sulfur concentration but it was not the aim of the present work.

Regarding final pH on this experimentation, Figure 5 shows that AZCT-M125-5, AZCT-M125-6, and AZCT-M125-7 at different sulfur concentrations decreased initial pH up to values within 0.63 and which depended of the sulfur concentration, that is, in general at higher sulfur concentration lower pH values except for AZCT-M125-7 where lower pH values were almost constant for all sulfur concentrations. For *Acidithiobacillus thiooxidans* 53987, pH value decreases within 1.68 to 1.23, depending sulfur concentration. It is important to observe that for other treatments, pH increases due to the addition of higher amounts of sulfur. According to the literature, to oxidize sulfur, bacteria are adsorbed to the insoluble sulfur particles, which increment the oxidation rate and acidification by sulfur-oxidizing bacteria. It has been reported that at concentrations within 0.5 to 5 g L^{-1} the elemental sulfur increases the oxidation rate, reduces pH, and produces sulfate,[36] and increasing elemental sulfur from 10 g L^{-1} to 20 g L^{-1} in a bioleaching process changed the amount of metals leached, *Thiobacillus thiooxidans* oxidizes 1 % of elemental sulfur,[7] *Thiobacillus* sp. ASWW-2 oxidizes 5 to 50 g L^{-1},[37] and *Acidithiobacillus ferrooxidans* oxidized 1 to 20 g L^{-1} of elemental sulfur when it was supplemented as a substrate.[38] Summarizing, available reports mention concentrations lower than those assayed in

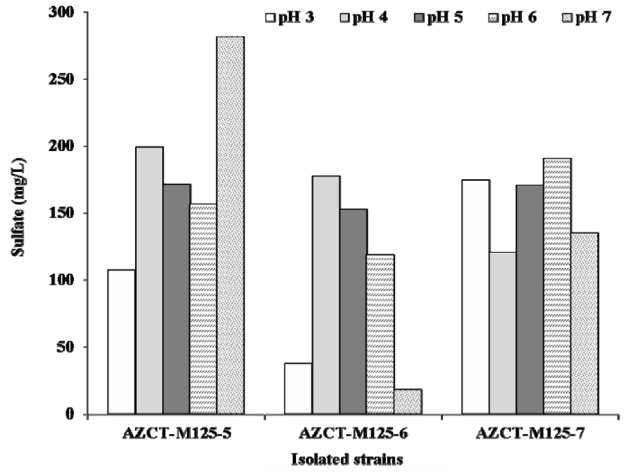


F i g . 5 – *Evaluation of pH during the sulfur oxidation by isolates at different sulfur concentrations after 7 days of incubation at 30°C, 140 rpm in modified Starkey medium*

our study. So, strains obtained in the present study that grow adequately at higher sulfur concentrations, such as 9 % (90 g L^{-1}), could be considered as possible candidates for the treatment of industrial wastes containing high sulfur concentrations such as spent catalysts coming from Claus process.

Evaluation of sulfur-oxidizing activity of isolates at different pH values

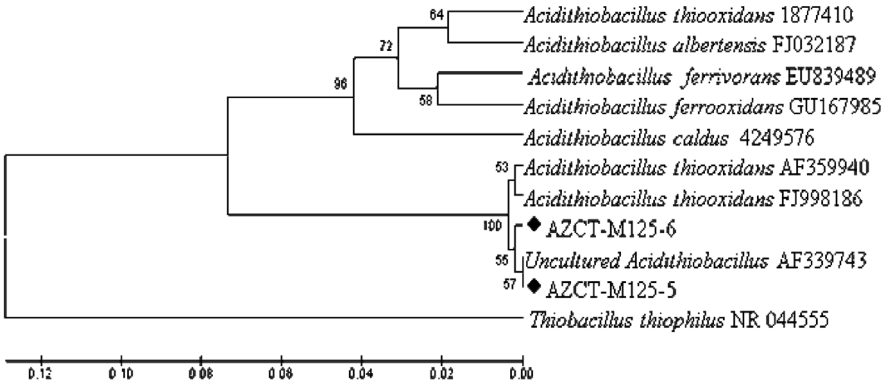
To know the influence of initial pH on the sulfur oxidation, only three isolates with the ability to produce sulfate at a wide range of sulfur concentrations were used (AZCT-M125-5, AZCT-M125-6 and AZCT-M125-7). Figure 6 shows that the three isolates were able to produce sulfate at different pH but at different extent. Strains presented a different sulfur oxidation behavior respect to the initial pH. Culture AZCT-M125-5 showed a high sulfate production at pH 7, reaching a production of 280 mg L^{-1}, AZCT-M125-6 produced 180 mg L^{-1} at pH 4, and AZCT-M125-7 at pH 6 had a production of 190 mg L^{-1}. It is noteworthy to mention that, in all cases, nevertheless of the assayed microbial culture the initial pH decreased up to pH values within 1.2 to 1.7 (data not shown). The sulfur oxidation activity of microorganisms was similar to strain *Thiobacillus* sp ASWW-2, which is able to oxidize sulfur in the range from pH 2 to 8, with a maximum oxidation at pH 4.[37] *T. thioparus* is able to grow within a range from pH 5 to 9, with an optimum close to pH 7.[32] For the case of acidophilic sulfur-oxidizing bacteria, a good growth at pH within 2.5 to 6.5 has been observed[20,22,39] with an optimum pH 4 for sulfur oxidation;[39] however, some acidophilic strains can grow at values lower than pH 3.[22] Based on this behavior, sulfur-oxidizing bacteria in the present study can be classified as acidophilic sulfur-oxidizing microorganisms.[40] Cultures coded as AZCT-M125-5, AZCT-M125-6, and AZCT-M125-7 can be classified as acidophilic sulfur-oxidizing, hence, they can be used in the leaching of heavy metals such as copper, nickel, cobalt, zinc, and molybdenum,[17,41,42,43] in the desulfurization of charcoal,[38] or for sulfur elimination from polluted samples such as spent catalysts that came from the Claus process.



F i g . 6 – *Effect of pH on sulfur-oxidizing activity of isolates after 7 days of incubation at 30°C, 140 rpm in modified Starkey medium*

Identification of best sulfur-oxidizing microorganisms

The resulting sequences of the strains coded as AZCT-M125-5 and AZCT-M125-6 were manually edited with the Bioedit Equence aligment editor and afterwards submitted to a search of similar sequences in the GenBank by means of the BLASTN program in the web page of NCBI (http://www.ncbi.nlm.nih.gov/). From the provided information of sequences, it was possible to taxonomically locate the cultures as follows: AZCT-M125-5 and AZCT-M125-6 as *Acidithiobacillus thiooxidans* (Fig. 7). The strain coded as AZCT-M125-5 had a 99.2 % of identity with the *Acidithiobacillus thiooxidans* with a GenBank accession number FJ998186 while strain AZCT-M125-6 had an identity of 98.4 % with the same sequence. The two new sequences reported herein were deposited in the GenBank with accession numbers JX134585



F i g . 7 – *UPGMA phylogenetic tree of 16S rRNA gene sequences from cultures AZCT-M125-5 and AZCT-M125-6. Sequences were aligned with ClustalX, and distances were calculated with the Kimura 2-Parameter substitution model. The scale bar indicates nucleotide substitutions per site. Number at the branches indicates the bootstrap values of 1,000 resampling. Thiobacillusthiophilus served as out-group*

and JX134586. Finally, the strain coded AZCT--M125-7 has not been identified yet because direct sequencing was not possible, perhaps due to the presence of more than one bacterial species in the microbial culture.

Conclusions

It was possible to isolate a large number of microorganisms from environmental samples using different sulfur sources, nevertheless only three of them showed an outstanding ability to grow chemolithotrophically and oxidize sulfur at concentrations above 1 and up to 9 % (w/v) and pH within 3 to 7. Two of them are closely related to *Acidithiobacillus thiooxidans*. Data suggest them as candidates for the treatment of industrial wastes containing sulfur concentrations as high as 9 % (w/v), however further studies using high sulfur-content industrial wastes such as spent catalysts are needed.

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