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Characterization and activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage decontamination

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ABSTRACT

Biological treatment with sulphate-reducing bacteria (SRB) has been considered as the most promising alternative for acid mine drainage (AMD) decontamination. Normally, these wastewaters contain high concentrations of sulphate and heavy metals, so the search for SRB highly resistant to metals is extremely important for the development of a bioremediation technology. A SRB consortium resistant to high concentrations of heavy metals (Fe, Cu and Zn), similar to those typically present in AMD, was obtained among several environmental samples, from a wastewater treatment plant. The phylogenetic analysis of the *dsr* gene sequence revealed that this consortium contains species of SRB affiliated to *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis*. The results show that the presence of usually lethal concentrations of Fe (400 mg/L), Zn (150 mg/L) and Cu (80 mg/L) is not toxic for the sulphate-reducing bacteria present in this sample. As a consequence, a very good efficiency in terms of sulphate reduction and metals removal was obtained. Both ethanol and lactate can be used by this inoculum as carbon source. With the other samples tested sulphate reduction was inhibited by the presence of copper and zinc. This highly metal resistant consortium will be used to inoculate a bioreactor to carry out AMD decontamination.

1. Introduction

Sulphate-reducing bacteria (SRB) are important members of microbial communities with economic, environmental and biotechnological interest. They can exist in a variety of environments such as soils, sediments and domestic, industrial and mining wastewaters [1]. SRB are included in a group of chemoorganotrophic and strictly anaerobic bacteria, which contains representatives of the genera *Desulfovibrio*, *Desulfomicrobium*, *Desulfobacter* and *Desulfotomaculum,* among others [2].

SRB have the ability to reduce sulphate to sulphide and this sulphide reacts with certain metals dissolved, such as copper, iron and zinc, forming insoluble precipitates [3]. Anaerobic reduction of sulphate by SRB has been reported to be used for the treatment of a variety of sulphate-containing industrial effluents [4–6], being mining wastewaters, rich in heavy metals, one of the most relevant examples. However, the use of SRB for these applications has generally one important limitation: lack of bacterial resistance to metals.

Heavy metals are generally toxic for microorganisms, including SRB, due to substitution of essential ions on cellular sites, and blockage of functional groups of important molecules such as enzymes. This results in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity [7,8]. It has been reported that toxic concentrations of heavy metals for SRB range from a few ppm to as much as 100 ppm [7,8]. The metal resistance of SRB varies with the species. Different organisms exhibit diverse responses to toxic ions, which confer them a certain tolerance to metals. They have a number of specific resistance mechanisms, such as sequestration or transformation to other chemical species [9].

The search for SRB resistant to metals is very important for the development of efficient bioremediation processes based on the use of these bacteria.

The purpose of this work was to compare the tolerance of SRB consortia from several environmental sources to the most concentrated heavy metals (Fe, Zn and Cu) present in acid mine drainage (AMD) of S. Domingos mine (an abandoned copper mine in South-east Portugal).

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The AMD from S. Domingos pit lake is highly acidic (pH around 2) and characterized by high concentrations of heavy metals mainly Fe (500 mg/L), Cu (50 mg/L), Zn (110 mg/L) and sulphate (3100 mg/L) [10,11].

This work is part of a wider research programme in which the main goal is the design of a sulphate-reducing bacteria technology for the decontamination of AMD from S. Domingos mine. Therefore, the results obtained in these studies will be taken into account for the subsequent development of a sulphate-reducing bioreactor to remove high levels of sulphates and heavy metals present in real contaminated AMD.

2. Materials and methods

2.1. Sampling and chemical characterization

The search for SRB was done by using environmental samples collected in the Provinces of Algarve and Alentejo, South Portugal. In Algarve, soil samples from Monchique thermal place, sediments from Formosa estuary and sludge from two wastewater treatment plants, located in Montenegro and in Estói, were collected. Sediments from the mining area of S. Domingos (Alentejo) were also collected in two places: Corta (near the top of the pit lake) and near the stream of Chança. The samples were identified by the names of the places where they were collected. The use of natural sources has advantages over the use of pure bacterial cultures: they contain bacterial consortia that facilitate the development of reducing conditions and they are also more easily available [12].

Multielemental analysis of the environmental samples collected (Table 1) was carried out by Total Reflection X-Ray Fluorescence (TXRF) using an EXTRA-IIA (Atomika Instruments) spectrometer. Previous to instrumental analysis, samples were submitted to microwave acid digestion in closed Teflon® Parr® bombs, using *aqua regia*, HF and gallium as internal standard [13].

2.2. SRB enumeration

SRB populations were enumerated by the three-tube Most Probable Number (MPN) assay with serial dilutions in Postgate E medium [14]. The experiment was performed in triplicate. The MPN tubes were incubated at room temperature (±21 °C) for 5 days.

2.3. Batch experiments

Experiments were performed in batch and in anaerobic conditions. All experiments were performed in duplicate using 120 mL glass bottles containing 100 mL of growth medium with pH around 7. Oxygen diffusion was eliminated by the addition of 10 mL of sterile liquid paraffin. After inoculation, the bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (±21 °C).

The growth media used were modifications of the Postgate B medium [14]. The modifications of the medium composition for individual experiments are described below.

2.3.1. Study of efficiency of sulphate reduction by SRB populations from the collected samples

The growth medium used was supplemented with resazurin as redox indicator (0.03 g/L). The inoculation was carried out using 5 g of each of the samples previously mentioned (sediment, sludge or soil).

Further tests were carried out using bacterial cells collected from the previous study. The bacterial cells were harvested by centrifugation, washed and transferred to the test solutions.

2.3.2. Effect of heavy metals on sulphate reduction

The growth media contain lactate (6 g/L) as carbon and energy source, sulphates (3.5 g/L) and resazurin as redox indicator (0.03 g/L). Different experiments were carried out with 0.4 g/L or 0.8 g/L of iron only, or 0.4 g/L of iron, 0.08 g/L of copper and 0.15 g/L of zinc in the mixture. The metal salts used for the study were $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$ and $CuSO_4{\cdot}5H_2O$. A test without metals was done as control.

2.3.3. Effect of carbon source on sulphate reduction

The growth media contained a carbon and energy source (6 g/L) and resazurin as redox indicator (0.03 g/L). Lactose and ethanol were the carbon sources selected, considering the great availability of these compounds in by-products of the cheese and winery Portuguese industries and the eventual future utilization of these wastes as carbon sources. Lactate is the carbon source most widely used by SRB [14,15] and was also used as control.

Table 1
Elemental composition of the environmental samples used for SRB search. Values for the metals tested in the present study are in bold.

Element	Concentration (g/kg)					
	Monchique	Montenegro	Corta	Formosa	Estói	Chança
K	30	7.6	14	11	2.3	14
Ca	4.8	43	4.7	12	77	0.52
Ti	6.8	2.8	5.3	2.2	1.5	3.8
V	0.178	0.052	0.147	0.068	0.052	0.099
Cr	<0.03	0.040	0.065	0.024	0.029	0.054
Mn	0.83	0.13	<0.03	0.075	0.18	<0.03
Fe	**24**	**16**	**73**	**15**	**8.4**	**61**
Ni	<0.01	0.018	<0.01	<0.01	0.022	<0.01
Cu	**0.009**	**0.10**	**2.0**	**0.009**	**0.25**	**0.72**
Zn	**0.077**	**0.430**	**0.99**	**0.061**	**1.2**	**0.55**
As	<0.01	<0.01	1.0	0.011	<0.01	1.6
Se	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007
Rb	0.25	0.068	0.098	0.085	0.017	0.074
Sr	0.82	0.118	0.145	0.079	0.36	0.094
Cd	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Sn	<0.04	<0.04	3.2	<0.04	<0.04	0.16
Sb	<0.04	<0.04	0.36	<0.04	<0.04	0.19
Ba	0.7	0.4	0.6	<0.2	0.4	0.5
Pb	0.020	0.08	6.0	0.019	0.10	4.6

2.4. Sampling and analytical methods

Periodically, 5 mL samples were collected using a syringe, and filtered using a 0.2 μm filter. pH, redox potential, and soluble concentrations of lactate, lactose, ethanol, acetate, sulphate and heavy metals were measured in each sample. Redox potential and pH were measured immediately after sample collection and filtration using a pH/E Meter (GLP 21, Crison). A high performance liquid chromatograph (Beckman), equipped with a polyspher® OAHY column (30 cm × 0.65 cm, Merck) and a RI detector, were used for lactate, lactose, ethanol and acetate analysis. Sulphate concentration was measured by a spectrophotometer (Hach-Lange DR2800) using the method of sulfaVer4 (Hach-Lange). For determination of the dissolved metals (Fe, Cu and Zn) the filtered samples were acidified with nitric acid and analysed by Atomic Absorption Spectroscopy (AAS) using a Shimadzu, AA-680 model spectrometer.

An optical microscope equipped with a digital camera (Leica D C300FX) was used to visualise the bacteria present in the inoculum of Montenegro. Cells were centrifuged (10 min at 4000 rpm) and washed with sterile distilled water prior to Gram staining.

2.5. Molecular characterization

2.5.1. Extraction of DNA

Total genomic DNA was extracted from cell cultures grown on modified Postgate B media. The cells were harvested from 20 mL of cell culture by centrifugation at 4000 rpm for 10 min and twice washed with chilled deionised water. DNA extraction was carried out by the following method: 300 μL of SDS lysis mixture (500 mM TrisHCl pH 8, 3% SDS, 100 mM NaCl) and 300 μL of phosphate buffer pH 8 were added, followed by a freeze–thaw treatment (three cycles consisting of 1 min in liquid N_2 followed by 5 min in a 37 °C water bath). After cellular lysis, 300 μL of chloroform-isoamyl alcohol (24:1) were added. The solution obtained was centrifuged at 13000 rpm, for 10 min. After precipitation with isopropanol at −20 °C, for 20 min, DNA was resuspended in 35 μL H_2O. Nucleic acid extraction was evaluated on a 1% (w/v) agarose gel electrophoresis in Tris–Acetate–EDTA (TAE) buffer.

2.5.2. PCR amplification of dsr gene

PCR was conducted in a total volume of 50 μL. Community dissimilatory sulphite reductase (*dsr*) genes were amplified using the primers DSR1F and DSR4R [1,16,17], which amplify a 1.9 kb fragment. The primers were purchased from Thermo Ficher Scientific. The reaction mixture used for PCR amplification contained 30.75 μL of H_2O, 1 μL of each primer (10 pmol/μL), 1 μL of dNTPs (10 mM), 5 μL of $MgCl_2$ (25 mM), 10 μL of 5× Go Taq® buffer (Promega, Madison, USA), 0.25 μL of GoTaq® DNA polymerase (Promega, Madison, USA), and 1 μL of DNA. The DNA of a strain of *Desulfovibrio* subsp. and of *Escherichia coli* were used as positive and negative controls, respectively. PCR amplification was carried out in a thermocycler (T1, Biometra). Thermal cycling was carried out by using an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min and completed with an extension period of 5 min at 72 °C. The PCR products were separated in a 1% (w/v) agarose gel in TAE buffer.

2.5.3. Cloning of dsr gene and RFLP analysis

PCR products were purified (E.Z.N.A.™ Gel Extraction Kit, Omega) and ligated into the cloning vector pGEM®-T Easy followed by transformation into competent host cells, according to the manufacturer's instructions (Promega, Madison, USA). Individual colonies were screened by direct PCR amplifications with the DSR1F and DSR4R primers according to the conditions described above. RFLP analyses were done using the restriction enzymes HhaI and HaeIII (Promega). Fragments of the digested PCR products were separated in a 2% (w/v) TAE agarose gel.

2.5.4. Sequencing and phylogenetic analysis

Representative clones from each digestion pattern were selected for sequencing at CCMAR (Centro de Ciências do Mar, Universidade do Algarve). The PCR fragments were amplified and purified. Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic trees were constructed using MEGA 4 and the Neighborhood-Joining algorithm was applied.

3. Results

3.1. Determination of sulphate-reducing bacteria population

Large number of SRB was observed in the sludge coming from the wastewater treatment plants of Montenegro and Estói where 1.8×10^7 CFU/g and 5.3×10^6 CFU/g, respectively, were found (Fig. 1). In the soil samples of Monchique thermal place, the number of SRB reached was 2.3×10^4 CFU/g, while in the sediments of Formosa estuary 5×10^3 CFU/g were found. In both samples from the mining area (Corta and Chança) SRB were not detected.

The efficiency of sulphate reduction by SRB existing in the samples collected was investigated. After 42 days of study, the most efficient sulphate reduction was verified with the bacteria present in the samples from Montenegro (99.5%) and Estói (93.0%). Monchique presented a sulphate reduction efficiency of 64.7% and Formosa 33.5%. Sulphate reduction was not observed in the samples of the mining area (Corta and Chança).

The following studies were performed using the inocula of Montenegro, Estói and Monchique, which presented the highest efficiency in terms of sulphate reduction (higher than 50%) in the batch tests.

3.2. Effect of heavy metals on sulphate reduction

In these experiments the efficiency of biological sulphate reduction in the presence of two different concentrations of iron and in the presence of iron, copper and zinc was investigated. The effect of those metals on sulphate reduction is shown in Fig. 2. The presence of iron in the medium generally affects the rate of sulphate

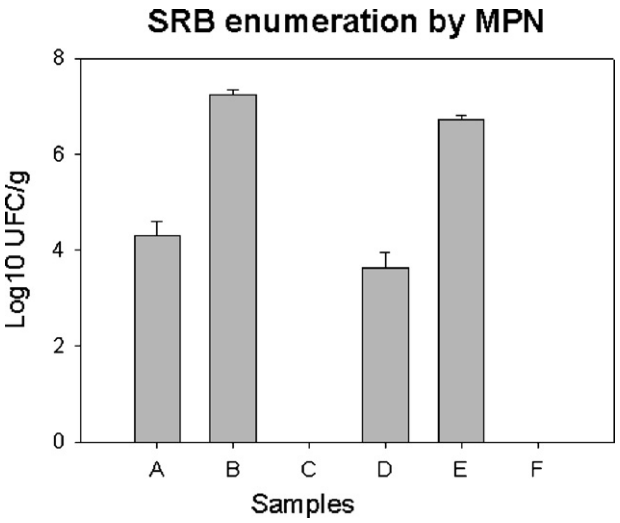

Fig. 1. SRB enumeration in samples collected. Samples: A, Monchique; B, Montenegro; C, Corta; D, Formosa; E, Estói; F, Chança. Data are the average of triplicates and error bars indicate the standard deviations.

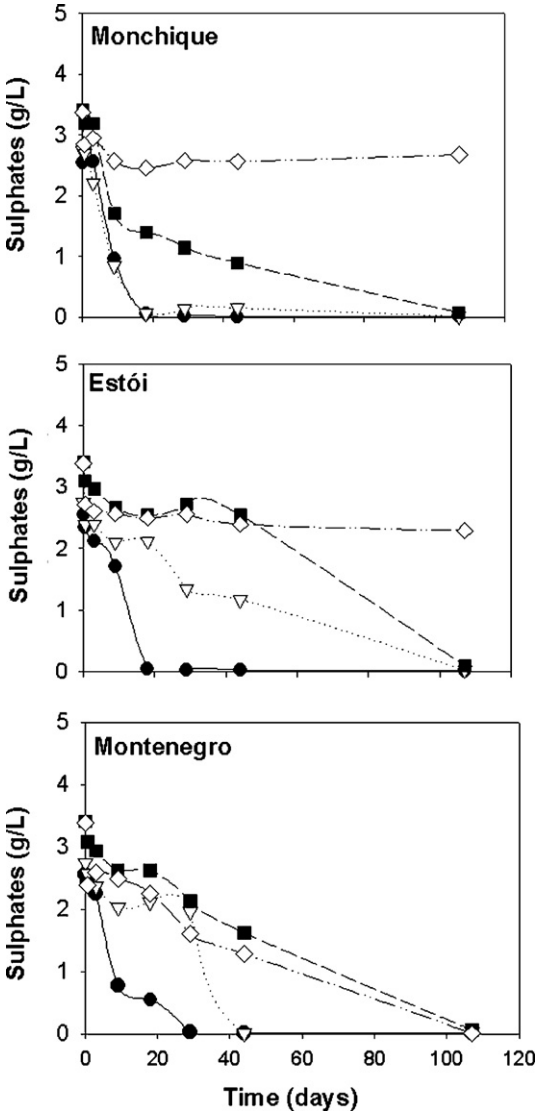

Fig. 2. Evolution of sulphate reduction by SRB in the different tests: without metals (●), 0.4 g/L Fe (▽), 0.8 g/L Fe (■) and mix test with Fe, Cu and Zn (◊). Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown.

reduction, and this also depends on iron concentration. The only exception was observed with the sample of Monchique, for which 0.4 g/L of iron in the medium did not influence the efficiency of sulphate reduction as a function of time: both in the absence and in the presence of that concentration of iron, sulphate was completely reduced after 20 days of experiment. When the concentration of iron in the medium was increased to 0.8 g/L, sulphate concentration was reduced to less than half the initial value within 20 days.

According to these results (Fig. 2) it is observed that the presence of zinc and copper in the medium inhibits sulphate reduction by SRB in the samples of Monchique and Estói. However, this did not happen with the SRB from Montenegro. In fact, after 30 days of experiment sulphate concentration decreased about 50%, although the lag time was increased. Complete sulphate reduction was achieved after 110 days, showing that the toxic effect of these metals did not prevent sulphate reduction by this community.

The removal of these metals is an indirect consequence of biological activity, so a larger extent of reduction of sulphate to sulphide results in larger efficiency of metal removal.

The rate of decrease in concentration of copper in the medium was faster than that of zinc or iron. As shown in Fig. 3, with 80 mg/L Cu as initial concentration, this metal was completely removed from the medium within 15 days. This was observed with all the inoculum sources. In the case of zinc, the concentration decreased from 150 mg/L to 2.0 mg/L within 18 days, with SRB from Montenegro. With the other two inocula (Estói and Monchique), zinc was almost completely removed but after a much longer time (40 days). The removal of iron takes longer: this metal was significantly removed within 44 days (from 400 mg/L to 58 mg/L) with SRB from Montenegro, independently of the presence of zinc and copper in the medium (Fig. 4). Due to near neutral pH of the media, some precipitation of those metals as (oxy)hydroxides probably occurs, as already reported by Zagury et al. [16]. This is particularly consistent with a certain decrease in iron concentration almost immediately after the beginning of the experiments (Fig. 4).

According to the results obtained the sample of Montenegro contains the SRB most resistant to the metals under study. This inoculum is constituted mainly by Gram-negative bacilli and some cocci (Fig. 5).

3.3. Effect of carbon source on sulphate reduction

Experiments were conducted to compare the sulphate reduction profile in the presence of the three carbon sources mentioned (Fig. 6). The most efficient sulphate reduction was generally observed with lactate for all inocula. Lactate was totally consumed in the first days of experiment at the same time as acetate production was observed.

Efficient sulphate reduction by SRB with ethanol as carbon source was only observed with the inocula from both wastewater treatment plants (Montenegro and Estói). Although the concen-

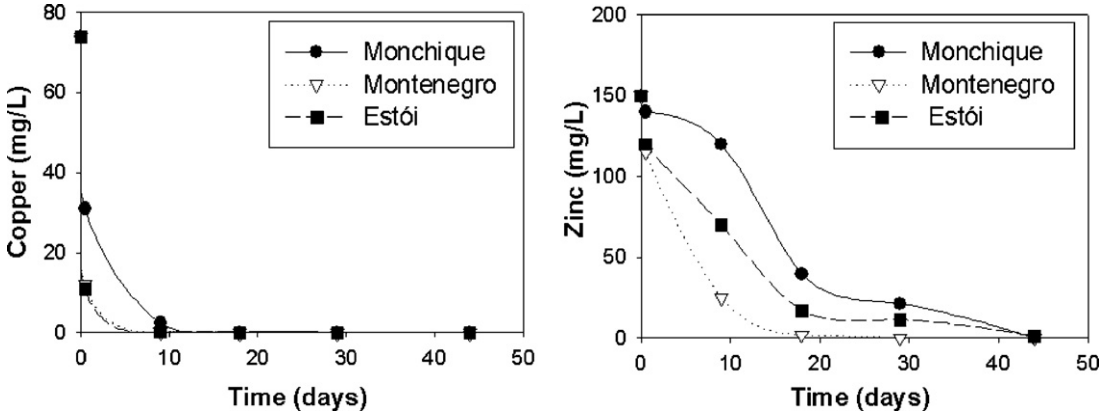

Fig. 3. Evolution of copper and zinc concentrations in the media as a function of time. Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown.

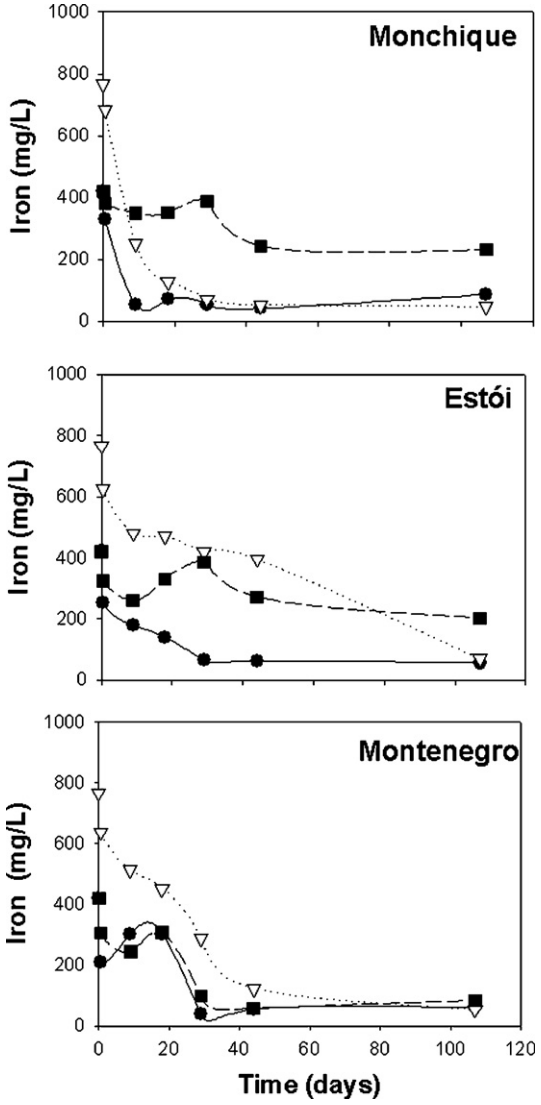

Fig. 4. Evolution of iron concentration in the media as a function of time for the different tests: 0.4 g/L Fe (●), 0.8 g/L Fe (▽) and mix test with Fe, Cu and Zn (■). Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown.

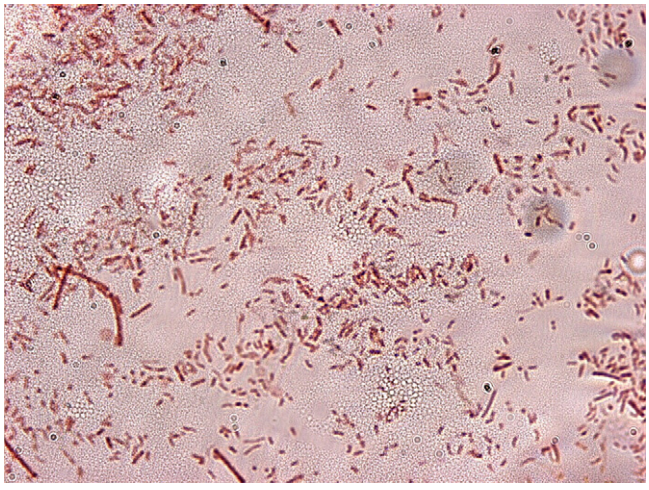

Fig. 5. Photomicrograph of the bacteria present in the inoculum of Montenegro. Amplification of 1000×.

tration of ethanol has decreased in the sample of Monchique, no sulphate reduction was observed.

In the presence of lactose, a more complex organic molecule, no sulphate reduction was observed independently of the inocula. However, lactose consumption was detected, which can be due to the presence of bacteria other than SRB in the consortium, which are able to use this carbon source in their metabolism.

3.4. RFLP and sequence analysis

The dissimilatory sulphite reductase gene (*dsr*) was used to elucidate the composition of the SRB consortium of each of the different samples investigated. To achieve this goal, the primer pair DSR1F/DSR4R [1,17,18] which amplifies a 1.9 kb *dsr* gene fragment was used. These primers have shown to be a powerful tool on SRB diversity studies where the phylogenetic analyses were based either on restriction analysis of the cloned fragment or sequencing the cloned 1.9 kb *dsr* fragment [19–21].

A total of 43 clones were obtained: 8 from the samples of Monchique (Monc), 11 from Estói (E) and 24 clones from Montenegro (Mont) samples. The combination of the RFLP patterns from both enzymes produced four patterns for Monchique samples, five for Estói and seven for Montenegro samples. The sequences obtained for the *dsr* gene from these clones were analysed and equally produced four major clusters (Fig. 7). The 32 sequences obtained were registered in the GenBank (EU189153–EU189184). The first cluster is formed by Mont25, Mont26, Mont27 and Mont10 clones and the sequence analysis revealed that these clones share high similarity with *Desulfovibrio desulfuricans* strain F28-1 as the most closely related cultured species. The second cluster is constituted by the clones Mont3, Mont23 and E3 and the cultured species most closely related to them is *D. desulfuricans* AF273034.1. The remaining clones from Estói constitute a third group, in which E4 is the most distant. By sequence analysis the E1 clone is affiliated to uncultured bacterium clone NTUA-1A-DSR14 EF645665.1, whereas the clones E2 and E7 share similarity to uncultured bacterium clone NTUA-1A-DSR1 EF645664.1. The sequence of clone E4 is affiliated to uncultured SRB clone GranDSR2 and the cultured *D. desulfuricans* isolate SRDQC. The cluster constituted by Monc1, Monc3 and Monc4 clones showed similarity with the cultured *Desulfovibrio fructosovorans*, namely the sequence of Monc 1 is close to *D. fructosovorans* AB061538.1 and Monc 3 and Monc 4 are related to *D. fructosovorans* DSM 3604 AF418187.1. Regarding the clone Monc 2, it is phylogenetically affiliated with *Desulfovibrio vulgaris oxamicus*. The sequence analysis of the clone Mont 22 revealed affiliation to uncultured bacterium clone, NTUA-1A-DSR1 EF645664.1 and to cultured *Desulfobulbus rhabdoformis* AJ250473.1.

4. Discussion

Regarding the diversity of samples it was expected to obtain diverse bacterial communities, in order to increase the probability of finding metals resistant SRB suitable to be used with the aim of AMD bioremediation. The highest efficiency of sulphate reduction by SRB was observed in sludge samples from the wastewater treatment plants of Montenegro and Estói, where larger numbers of SRB were detected. The presence of SRB was not observed in the samples from the mining area (Corta and Chança). Accordingly, sulphate reduction was not detected either. A possible reason for this result is the high acidity of these sediments, which do not allow SRB survival. In the remaining experiments the pH value was maintained at about 7, much higher than the pH values of the media with Corta or Chança samples, which decreased from 6.7 to 3.7 and 4.5, respectively, immediately after addition of the solid samples. The difficulty to grow SRB in acid medium has already been mentioned in the literature by Garcia et al. [22].

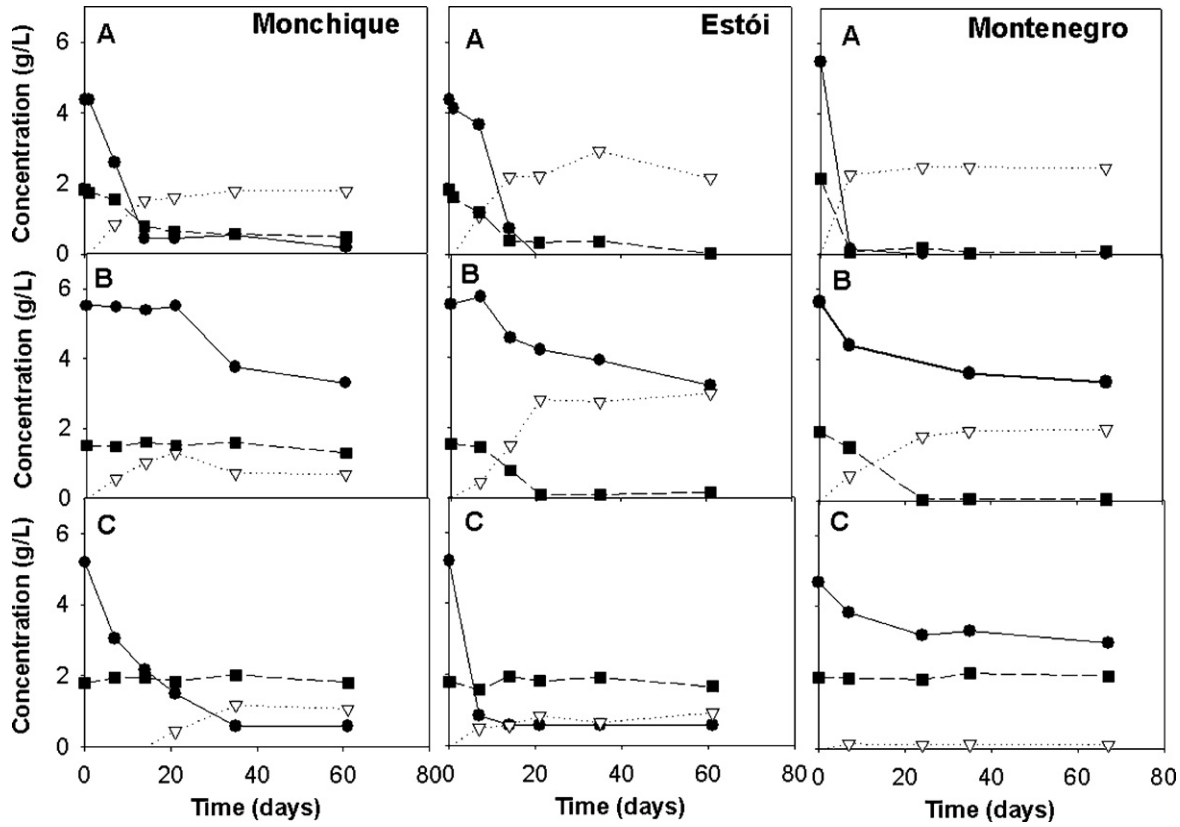

Fig. 6. Evolution of lactate, acetate, ethanol, lactose and sulphate concentrations in the presence of lactate (A), ethanol (B) and lactose (C) as carbon source on sulphate reduction by SRB. Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown. Symbols: (●) carbon source, (▽) acetate and (■) sulphates.

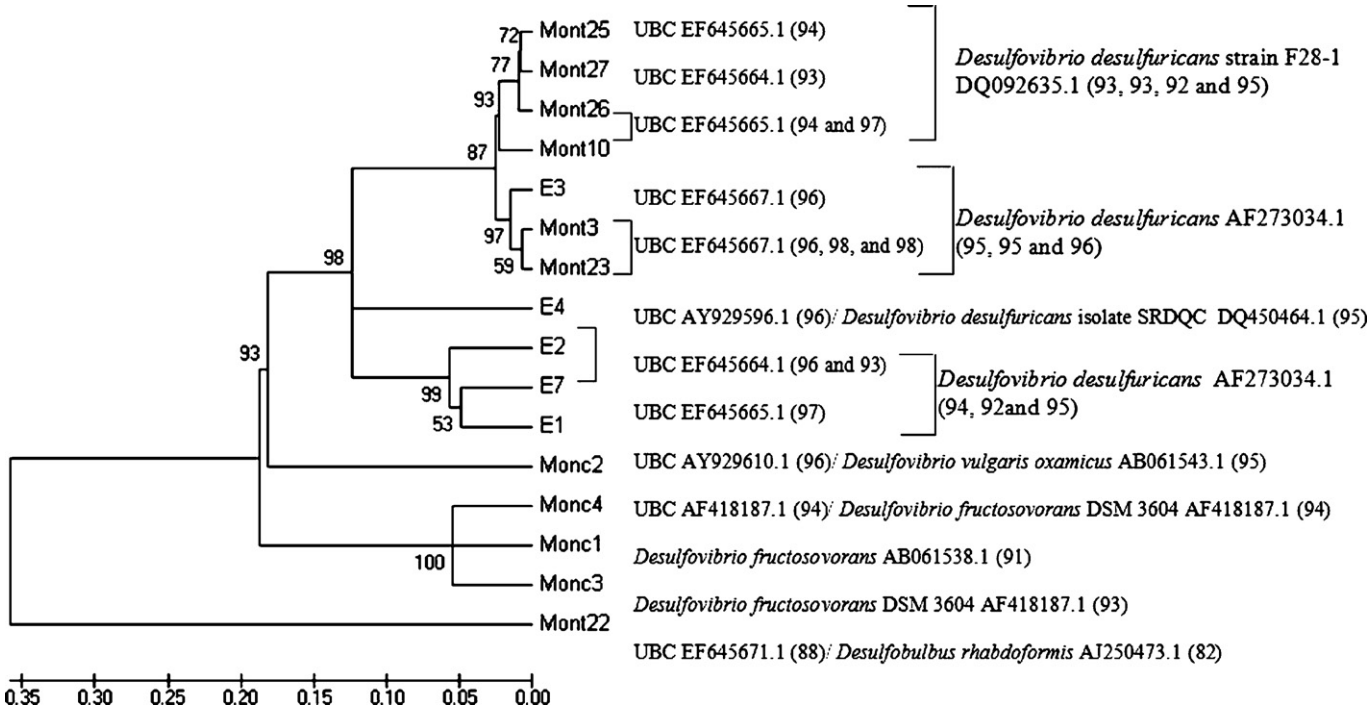

Fig. 7. Phylogenetic tree constructed for clones from Monchique (Monc), Estói (E) and Montenegro (Mont) samples using *dsr* gene. The Neighborhood-Joining algorithm was used. Bootstrap values are indicated on branches. Following the clone name the most closely related species and the most related cultured species are indicated. The percentage of similarity is indicated in brackets. UBC—uncultured bacterium clone.

Table 2
Toxic concentrations of metals reported by other authors.

Metal	Toxic concentrations (mg/L)	SRB cultures	Reference
Cu (II)	>10	*Desulfomicrobium* sp.	Azabou et al. [26]
	9	*Desulfovibrio vulgaris* and *Desulfovibrio* sp.	Cabrera et al. [7]
	2	*Desulfovibrio desulfuricans* G20	Sani et al. [8]
	12	Mixed culture	Utgikar et al. [25]
	4–20	Mixed culture	Hao et al. [23]
Zn (II)	>125	*Desulfomicrobium* sp.	Azabou et al. [26]
	20	*Desulfovibrio vulgaris*	Cabrera et al. [7]
	20	Mixed culture	Utgikar et al. [25]
	13	*Desulfovibrio desulfuricans*	Poulson et al. [24]
	25–40	Mixed culture	Hao et al. [23]
Fe (III)	>60	*Desulfomicrobium* sp.	Azabou et al. [26]

In this study the SRB existing in the consortium from Monchique demonstrate a certain tolerance for iron. This behaviour can eventually be due to the fact that among the three samples tested, the sample from Monchique is the one that presents the highest iron content (Table 1) and therefore its SRB are probably more adapted to that metal. Our results show that the presence of zinc (150 mg/L) and copper (80 mg/L) significantly inhibited the activity of SRB present in the samples of Monchique and Estói. It has been reported that the characteristic toxic concentration of zinc for SRB is 13–40 mg/L [23–25]. It has been mentioned that SRB are sensitive to copper, with no growth observed at 2 mg/L [8] or 10 mg/L [26] of this metal. In the present study, even though the concentration of both elements was higher than those previously reported (Table 2), they did not stop sulphate reduction by SRB from Montenegro. The difference in behaviour can be due to a higher natural tolerance to these metals of the inoculum of Montenegro compared to the others and suggests different phylogenetic affinities.

In this study, the removal of metal was evaluated. It was observed that copper was the first element to be removed, followed by zinc and then iron. This result was in accordance with the literature [27] and can be explained by the solubilities of CuS, ZnS and FeS, which are respectively 5.83×10^{-18} mg/L, 2.31×10^{-7} mg/L and 3.43×10^{-5} mg/L [28,29]. Hence, copper needs the least amount of sulphide to precipitate, while iron needs the highest. In most cases, although the yield of sulphate reduction was more than enough to precipitate completely all the metals in the solution, the precipitation of iron was never quantitative. This is probably due to the fact that produced H_2S easily escapes as a gas during sampling being some of it not accessible to the dissolved metals. The formation of sulphur species with intermediate oxidation states [30] and/or the re-dissolution of $Fe(OH)_3$ in reducing conditions [16,29], may also be responsible for incomplete iron precipitation.

The phylogenetic tree showed that Montenegro is the most diverse sample, having species closely related to two different sequences of *Desulfovibrio desulfuricans* (AF273034.1 and F28-1 DQ092635.1) and one related to another SRB genus *Desulfobulbus rhabdoformis*, a genus not found in the other samples. This fact is particularly relevant due to the excellent performance of the Montenegro SRB consortium in the presence of iron, copper and zinc compared with the other inocula and considering their ability to use ethanol as carbon source. *D. rhabdoformis* was recently identified on bioreactors working in the presence of ethanol as carbon and energy source [31]. In addition, its ability to use a significant range of substrates, namely propionate, lactate, pyruvate, malate and fumarate is known [32]. The Monchique consortium includes species affiliated to *Desulfovibrio fructosovorans,* which is known to differ from all other described *Desulfovibrio* species by the ability to use fructose [33].

The SRB consortium of Monchique samples also includes a sulphate and nitrate reducing bacterium *D. vulgaris oxamicus*, that was reclassified by López-Cortés et al. [34] as *D. oxamicus* sp. nov. comb. nov. The type strain (DSM 1925^T) of *D. oxamicus* is known to be able to oxidize incompletely lactate and ethanol to acetate [34]. Thus, the presence of this species, together with other than SRB in the consortium, may explain the slight decrease of ethanol, accompanied by an ineffective sulphate reduction.

The SRB inoculum from Estói samples is affiliated mainly with the cultured species of *D. desulfuricans*, which showed a similar performance to Montenegro SRB consortium in terms of sulphate reduction, in the presence of ethanol. The occurrence of clones affiliated to *D. desulfuricans* in both samples of Montenegro and Estói is not surprising, as both are from wastewater treatment plants whereas Monchique inoculum was collected from a thermal place.

The results of this study emphasise that the composition of the inoculum can be determinant in the performance of sulphate-reducing systems for the treatment of acid mine drainage. Thus, the Montenegro consortium, constituted by clones affiliated to *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis,* seems to be the more suitable for an application to the treatment of AMD containing sulphates and metals (at least Fe, Zn and Cu).

Sulphate reduction was affected by the type of carbon source used. Most efficient sulphate reduction was observed with lactate. When lactose was added as carbon source no sulphate reduction was detected, probably due to the complexity of this molecule. These results are consistent with those previously reported in the literature [5,6,15,16] mentioning the preference of SRB for simple organic molecules, like lactate or ethanol, instead of complex molecules such as lactose.

In addition, the ability of the highly metal-tolerant SRB consortium to use ethanol as carbon source is a promising result considering an eventual utilization of ethanol-rich wastes, which are easily available in Portugal.

The Montenegro consortium will be used to inoculate a sulphate-reducing bioreactor for the decontamination of AMD from S. Domingos mine.

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