Isolation of mesophilic sulphate-reducing bacteria from a microbial community: comparative study of the effect of pH and dissolved heavy metals on the reduction of sulphate

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Abstract. Metallurgical processes and mining are the main source of heavy metal contamination of water sources, rivers and lakes. There are a large number of physicochemical processes that can be applied for the immobilization of heavy metals from a liquid matrix. However, many of them are not particularly desirable because their low selectivity and inefficiency when high volumes of low metal concentration liquids must be treated. In such conditions, alternative biological processes have shown to be more useful than traditional physicochemical processes. One of those processes, bioprecipitation of metal sulphides is relevant due to the possibility of forming stable solids (very low solubility) with small volumes compared with other solids. This process is mediated by a broad group of organisms called sulphate reducers that are able to catalyze, under anaerobic conditions, the reduction of sulphate with organic compounds as electron donors. In this paper, we study the effect of the presence of various heavy metals and the pH on the ability to reduce sulphate by sulphate-reducing bacteria. We compare the reduction of sulphate by a microbial community obtained from the effluent of a tannery with a strain isolated from that community. Our results showed that sulphate reduction was significantly affected by pH changes whereas the presence of heavy metals did not show a significant effect. In addition, metal precipitation by the isolated strain was similar than that produced by the community.

Introduction

Different anthropogenic activities release heavy metals into the environment generating serious problems for the environment and human life. Conventional technologies for removing metals from effluents (including precipitation, ion exchange and absorption) are expensive, with low selectivity, ineffective for removing metals especially at low concentration and in addition they can generate high solid sludge volume [1]. Bioremediation with biologically produced H_2S offers attractive advantages over the conventional treatment technologies: lower volumes of sludge are generated, the solubility of sulphides are several orders of magnitude lower than that of hydroxides and the precipitation is more effective at lower concentrations [2].

Sulphate-reducing bacteria (SRB) are heterotrophic microorganisms that use low molecular mass organic acids (such as lactic) and alcohols (such as ethanol) as the carbon/energy source. The organic substrate is oxidized either completely to CO_2 or to some intermediate compound such as acetic acid using sulphate as terminal electron acceptor, generating sulphide. The biologically produced H_2S reacts with metals and form insoluble metal sulphide precipitates [3].

The aim of this work was to use a 2_{III}^{7-3} fractionated factorial design to study the effects of different heavy metals (Cr(III), Cu(II), Co(II), Cd(II), Ni(II) and Zn(II)) and the initial pH on the growth of a mesophilic SRB isolated from an area exposed to toxic tannery wastes. The behaviour of a strain isolated from the original community was compared with that of the community.

Methods

Source of inocula. Sediment samples were taken from the effluent of a tannery in the area of the Rio Lujan, Argentina. Samples were used for enrichment of the microbial community and later isolation of mesophilic SRB.

Media and growth conditions. Postgate`s B liquid media [4] was used to enrichment and growth. Incubation was done at 30ºC at neutral pH in 120 ml hermetically stoppered serum bottles completely filled with medium. For the fractioned factorial design, a synthetic medium was used [5] (with sodium lactate 50% 8.0 ml/l). The pH of the medium was adjusted to defined values (pH 5 or 7) with NaOH or HCl solutions. 10 ml of inoculum was added to the culture bottles; then they were supplemented with the corresponding metal solutions. Anaerobic conditions were maintained by bubbling of sterile N_2. Cultures were incubated at 30ºC until no further sulphate consumption was detected.

Isolation. Mesophilic SRB were isolated in plates with Postgate`s C medium plus 2% agar [4]. The plates were placed in sealed jar with an anaerobic environment generator sachet (AnaeroGen AN35; OXOID, Cambridge, UK) and incubated at 30ºC. OXOID Anaerobic Indicator (BR55) was used to check anaerobic conditions.

Analytical methods and others determinations. Sulphate concentration was determined by turbidimetric method (barium chloride method) using a Beckman DU 640 spectrophotometer at 450 nm. The dissolved heavy metal concentrations were measured using an atomic absorption spectrophotometer, (AA-6650, Shimadzu, Japan). Prior to measurements, samples were filtered by 0.22 μm membranes. Bacterial morphology was observed by using a epifluorescence microscope (Leica DM2500, Germany) by 4′,6′-diamidino-2-phenylindole (DAPI) staining method. DAPI was used at a final concentration of 0.5 μg/ml in PBS.

Fractional factorial design 2_{III}^{7-3}. Fractional factorial design allows screening a relatively large number of factors in a relatively small number of experiments. To study the effects of several heavy metals and initial pH on the growth of SRB, we applied a fractional factorial design 2_{III}^{7-3}. Seven factors were used at two levels: pH (7.0 and 5.0), Cu (II) (4.8 and 0 ppm), Zn (II) (6.1 and 0 ppm), Cr (III) (3.7 and 0 ppm), Co (II) (5.0 and 0 ppm), Cd (II) (5.5 and 0 ppm) and Ni (II) (5.7 and 0 ppm). Table 1 shows the applied experimental matrix, where each row represents an experiment and each column represents an independent variable. The signs + and − represent the two different levels (high and low). In the design 2_{III}^{7-3}, the two-factor interactions are not confounded with the main effects and at least three two-factor interaction effects can be estimated [6]. The three two-factor interactions were named with letters A to G (Table 2).

Table 1. Experimental matrix for fractional factorial design 2_{III}^{7-3} for 7 factors

Run	pH	Cu	Zn	Cr	Co	Cd	Ni
1	-	-	-	-	+	+	+
2	-	-	+	+	-	-	+
3	+	-	-	+	+	-	-
4	+	+	+	+	+	+	+
5	+	-	+	-	-	+	-
6	-	+	-	+	-	+	-
7	-	+	+	-	+	-	-
8	+	+	-	-	-	-	+
9	+	+	+	+	-	-	-
10	+	+	-	-	+	+	-
11	-	+	+	-	-	+	+
12	-	-	-	-	-	-	-
13	-	+	-	+	+	-	+
14	+	-	+	-	+	-	+
15	+	-	-	+	-	+	+
16	-	-	+	+	+	+	-

Table 2. Three two-factor interaction effects that can be estimated

Three two-factor interaction effects	Two-factor interaction		
A	Cu(II) − Ni(II)	Zn(II) − Cd(II)	Cr(III) − Co(II)
B	pH − Ni(II)	Zn(II) − Co(II)	Cr(III) − Cd(II)
C	pH − Cd(II)	Cu(II) − Co(II)	Cr(III) − Ni(II)
D	pH − Co(II)	Cu(II) − Cd(II)	Zn(II) − Ni(II)
E	pH − Cr(III)	Cu(II) − Zn(II)	Cd(II) − Ni(II)
F	pH − Zn(II)	Cu(II) − Cr(III)	Zn(II) − Co(II)
G	pH − Cu(II)	Zn(II) − Cr(III)	Co(II) − Cd(II)

Analysis of fractional factorial design. The effect of each variable x_i can be estimated from $Ex_i = (\Sigma\ y_{xi}^{+} - \Sigma\ y_{xi}^{-})\ 2\ /\ N$, where $\Sigma\ y_{xi}^{+}$ and $\Sigma\ y_{xi}^{-}$ are the sums of the responses where factor x is at $+$ or $-$ level, respectively, and N is the number of design experiments. To calculate the standard error of the effects, three replicates at nominal levels were run containing half of high level concentration of each metal and pH 7.0. To identify significant effects, normal probability plots were used. Non-significant effects are normally distributed around zero. Factor effects that fall on a straight line in those plots are non-significant, while those deviating are considered significant. The Dong´s algorithm (margin of error ME) was used as statistical method to evaluate whether a given effect E_X is significant [7].

Results and Discussion

Isolation and microscope observation of a sulphate–reducing strain. After several enrichment cultures of the original sample, a strain of SRB was isolated from solid medium. Black colonies (due to the presence of FeS) were transfer to liquid Postgate´s B medium. Microscopic observations showed that cells seemed to be homogenous, non-motile bacillus; they were frequently observed in pairs. Gram stain was negative as is common for mesophilic SRB.

Sulphate reduction kinetics. Sulphate concentration as a function of time was compared for the original microbial community and the isolated strain in the presence of nominal level of metals and without metals. As it can be observed in figure 1, the microbial community reduces sulphate faster than the isolate strain. In both cases, the presence of heavy metals at nominal levels seems not exert a significant effect on the rate of sulphate reduction.
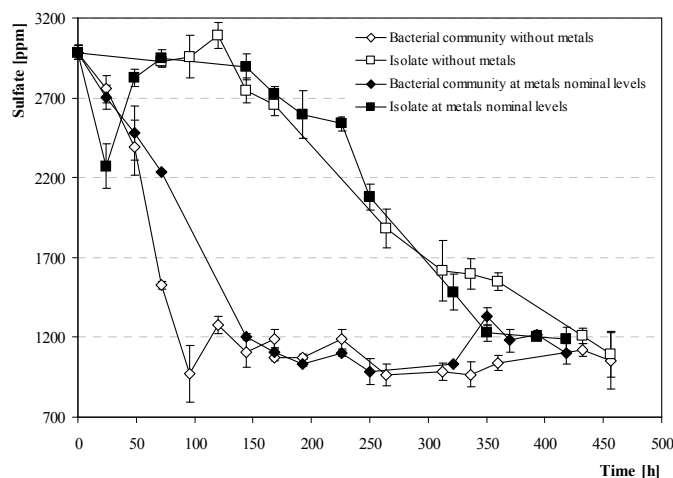

Fig. 1. Sulphate concentration vs. time for the bacterial community and the isolated strain in the presence and in the absence of heavy metals at nominal level
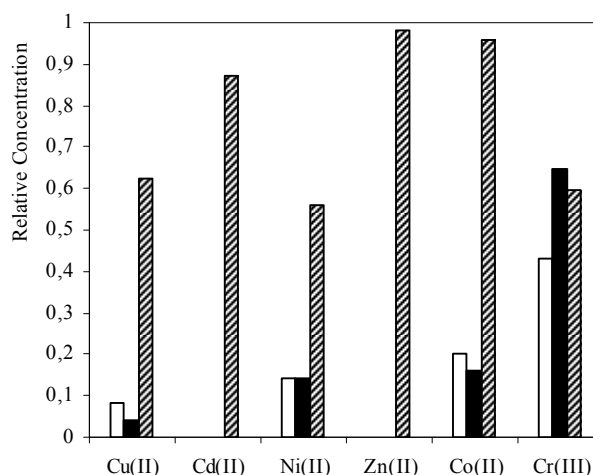
Fig. 2. Heavy metal Final Concentration/ initial concentration relation for the microbial community (White bars), strain (black bars) and abiotic control (dashed bars)

Heavy metal precipitation. To evaluate the efficacy of metal precipitation by the sulphate reducing bacteria, the concentration of heavy metals was measured at the beginning and at the end of the experiment in the three replicates at nominal levels (where all the metals were present). Figure 2 shows the percentage of metal precipitated in the replicates for the bacterial community, the strain and the abiotic control. As it can be seen, the biological production of sulphide promotes the precipitation of all metals. Cadmium and zinc are completely removed from the solution, copper, cobalt and nickel remain in low concentration and only about 50% of chromium is immobilized. Although the reduction of sulphate is faster by the community, the efficacy of metal precipitation of the isolated strain and the original community are similar at the end of the experiment.

Effect of the independient variables on the sulphate reduction. A normal probability plot was used to identify whether a variable (or an interaction) has a significant influence or not. Fig. 3 shows the normal probability plot obtained to analyze the influence of the seven independent factors and the seven three two-factor interactions on the sulphate reduction by the isolated strain. Dong´s algorithm was used to calculate the margin of error (*ME*) ($\alpha=0.05$). Effects exceeding *ME* are

significant. From this plot, it is clear that the pH, the presence of copper and the interaction *G* have a significant effect on sulphate reduction. While the presence of copper and factor *G* exert a negative influence on sulphate reduction, the increase in pH exerts an important positive influence.
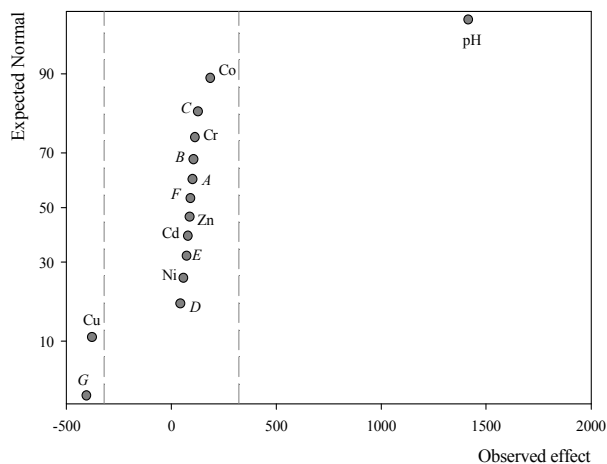
Fig. 3. Normal probability plot for the factors and interactions studied. Plot shows the normal distribution of each effect and the tree two factos interactions of tables 1 and 2 on the reduction of sulphate. Dashed lines represent the *ME*.

Similar analysis applied to the microbial community revealed that pH was the only factor with a significant effect, while the presence of heavy metals or their interactions have not shown any effect on the sulphate reduction. This might be associated with the presence in the original community of organisms with high resistance to copper and to the combined effect represented by interaction *G*.

Conclusions

Our experiments showed that the strain of SRB isolated from a community was able to growth in the presence of the heavy metals studied, although the reduction of sulphate was slower than the original microbial community. In both cases, slightly acidic pH values show a negative effect on sulphate reduction. Statistical analysis shows that cooper and the interaction between two factor *G* (which could be interaction between pH-Cu(II), Zn(II)-Cr(III) or Co(II)-Cd(II)) exert an inhibitory effect on sulphate reduction by the strain. A different experimental design has to be applied to distinguish between these effects.

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