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Fungal jarosite biomineralization in Río Tinto

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Abstract

Río Tinto is an extreme environment located at the core of the Iberian Pyrite Belt (IPB). It is an unusual ecosystem due to its size, constant acidic pH, high concentration of heavy metals and a high level of microbial diversity, mainly eukaryotic. Recently it was described the biomineralization of jarosite by *Purpureocillium lilacinum*, an acidic filamentous fungi isolated from the banks of the Tinto basin. In this study we further investigate the specificity of jarosite biomineralization by this fungi and its importance in the generation of jarosite in the Río Tinto basin. Our results clearly show that the ratio of the redox pairs and the Fe^{3+} concentration is important to achieve a specific biomineralization of jarosite. The amount of nucleation sites also seems to be critical, although the presence of nucleation sites by itself is not sufficient to precipitate jarosite. There is a good correlation between the sampling sites along the river in which hydronium-jarosite has been identified and the presence of *P. lilacinum*.

Keywords: Jarosite; Biomineralization; Río Tinto; Acidic fungi

1. Introduction

Extremophilic microorganisms capable of developing in extreme environments have attracted considerable attention because of the challenge that its discovery has posed to our current notion of limits of life and to their potential biotechnological applications [1]. Our research group has characterized the microbial diversity of an extreme acidic ecosystem, Río Tinto, a 92-km long acidic river (mean pH 2.3) containing a high concentration of toxic heavy metals in solution [2]. Río Tinto rises in Peña de Hierro, in the core of the Iberian Pyrite Belt (IPB) and flows into the Atlantic Ocean at Huelva. IPB is one of the largest massive sulfide deposits on Earth, formed as a hydrometallurgical deposit during the Paleozoic accretion of the Iberian Peninsula [3,4]. The river gives its name to an important mining district, which has been in operation for more than 5000 years. In the past, the extreme conditions of the river were considered the consequence of the intense mining activity in the area [5–8]. Today it is well established that the extreme conditions of the Tinto basin are the product of the metabolism of chemolithotrophic microorganisms, thriving in the high concentration of metal sulfides of the Iberian Pyrite Belt [9].

It is usually assumed that the toxicity of high metal concentrations in acidic habitats limits eukaryotic growth and diversity [10]. However, colored biofilms covering large surfaces of the Tinto basin as well as filamentous microbial communities are common features of this extreme environment [11–14]. In fact eukaryotic algae contribute over 60% of the river biomass [11]. The eukaryotic diversity in the ecosystem includes species of most of the major lineages [11,14–16]. Most of these microorganisms form complex photosynthetic biofilms, which differ in composition and structure along the physicochemical gradient of the river

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[16,17]. Among decomposers, fungi are the most abundant, and both unicellular and filamentous forms are present [11,18]. Fungi seem to play a fundamental role in the development of these biofilms.

The high concentration of heavy metals in solution results toxic to numerous aquatic organisms, therefore acidophilic fungi require suitable mechanisms to develop in these extreme conditions. At low pH, the cellular components must be adapted to the high concentration of extracellular acidity and be able to maintain the cytoplasm near neutrality using different homeostatic mechanisms [19,20]. In addition, heavy metals may induce denaturation of proteins, disruption of cellular membranes, act as antimetabolites of essential cellular functions and generate very reactive free radicals [21]. Thus, fungal survival and growth in these environments is possible through the development of several strategies including the synthesis of metallothioneins, extracellular precipitation, biosorption to cell walls, impermeability, and intracellular compartamentation among others [21–25]. The best understood mechanisms that fungi have developed to survive in the presence of toxic heavy metals are their sequestering on the cellular envelops or active transport to eliminate metals from the intracellular media. Moreover, it has been detected the capacity of different acidophilic fungi to specifically sequester toxic metals intracellularly, resulting the base of a methodology to eliminate and recover valuable metals from industrial contaminated wastewaters [26].

A recent characterization of the Río Tinto basin collecting samples from different stations along the river have rendered more than three hundred and fifty fungal isolates which have been identified by sequence analysis of their ITS regions. This sequence analysis revealed the Ascomycetes as the most abundant phylum. Basidiomycetes and Zygomycetes accounted for less than 2% of the sequenced isolates. Of the Ascomycetes, 52% clustered within the Eurotiomycetes class, while 27% grouped with the Dothideomycetes and 17% with the Sordariomycetes. The rest of the members of this phylum clustered with the Helotiales (2%), Mucorales (1%) and less than 1% with the Agaricomycetes (in preparation). Concerning metal tolerance Eurotiomycetes and Sordariomycetes isolates showed the highest resistance to toxic heavy metals, much higher than the concentrations detected in the river, while members of the Dothideomycetes showed a level of resistance to concentrations similar to those existing in the water column, and those from Basidiomycetes were in general less metal tolerant.

Testing the metal resistance profile of different fungal isolates from the Río Tinto basin the formation of a yellow-ochre precipitate, identified by XRD as hydronium-jarosite, was observed during the growth of *Purpureocillium lilacinum*, which was not generated during the growth in the same conditions of other acidophilic isolates belonging to the *Penicillium* and *Bispora* genera [27]. Non-viable cells were able to promote the formation of jarosite, although with much less efficiency. It was concluded from these results that *P. lilacinum* was able to specifically promote the biologically induced mineralization of jarosite [27]. In this study a deeper evaluation of the specificity and environmental conditions involved in the jarosite biomineralization by *P. lilacinum* is presented to ascertain its participation in the formation of jarosite in the Tinto basin.

2. Materials and methods

2.1. Fungal isolation and characterization

Samples of water and sediments from different sampling stations were collected and processed as described in Oggerin et al. (2013) [27]. All fungal isolates were classified according to their morphological characteristics and identified by ITS sequence comparison as described by Diaz et al. (2009) [28]. Forward and reverse sequences were assembled and edited using Geneious Pro 4.7.6 (Biomatters, New Zealand) and ITS consensus sequences were compared with the sequences from the GenBank database by using BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information). Phylogenetically related sequences were aligned using the CLUSTAL X (2.0) software [29] and alignments were trimmed using the Se–Al v2.0a11 Carbon software [30]. Cladograms of aligned sequences were obtained by maximum-parsimony analysis with the Heuristic Search algorithm implemented in PAUP v4.0a134 software [31] with 1000 replicates of random taxon addition and tree-bisection-reconection branch-swapping algorithm and with a statistical analysis by Bootstrab [32] based on 1000 resamplings.

2.2. Culture conditions

Fungal strains were cultured in Río Tinto waters collected from different sampling stations after their filtration through 0.22 μm pore sized membranes filters, with the addition of 0.5% glucose, 0.5% malt extract and 0.5% yeast extract. Cultures were incubated at 30 °C in flasks containing 100 ml of media in an orbital shaker at 120 rpm for one month. Precipitated minerals were collected, washed with distilled water and air-dried at 28 °C. Control experiments consisting in non inoculated culture media and media inoculated with autoclaved mycelium (non-viable cells) were also performed. All experiments were carried out in triplicate.

2.3. Analytical methods

Elemental composition of water samples and media were performed by ICP-MS and TXR as described in Oggerin et al. (2013) [27]. Purified biominerals were examined by XRD using a X'Pert Diffractometer (Panalytical, Netherlands) with a graphite monochromator and an automatic slit and were scanned continuously at $1°min^{-1}$ from 5° to 80° using a Cu Kα radiation source. Data were processed using the XPowder program for determination of mineral composition [33]. Scanning and transmission electron microscopy analysis were performed as described in Oggerin et al. (2013) [27]. Samples were also examined by optical microscopy by using an Axioskop microscope (Zeiss, Germany).

3. Results and discussion

To test the specificity of hydronium-jarosite generation with *P. lilacinum* we expanded the fungal acidophilic isolates tested using isolates from Río Tinto covering the main taxonomic groups identified in the river so far. Table 1 display the taxonomic adscription of the acidophilic isolates tested for the precipitation of jarosite. Members of the different fungal phyla: Ascomycota, Basidiomycota and Zygomycota have been tested. None of them with the exception of isolates from *P. lilacinum* were able to produce hydronium-jarosite during growth, which underlines the specificity for the biomineralization of jarosite described for this member of the order Hypocreales. Only one isolate with a high sequence ITS homology with *Verticillium leptobactum* produced a precipitate which correspond to a mixture of minerals, none of them jarosite, according to its XRD analysis. As previously shown the type strain from the DSMZ also exhibits this property, which indicates that specific structural features of the cell wall of this microorganism are responsible for the nucleation of this mineral. As already described, the non-viable cells can also promote the precipitation of jarosite although with much less efficiency, probably due to the lack of EPS, which allowed concluding that it corresponds to a biologically induced mineralization process (BIM) [27].

Previous reports have already suggested that nucleation and resultant mineral precipitation are influenced by factors such as pH, cell wall composition and cellular microenvironment [34]. To evaluate the capability of *P. lilacinum* to generate jarosite at the different ionic conditions existing along the course of the river, media prepared with water collected from different sampling stations were tested. Table 2 displays the different physico-chemical variables measured for these samples. Table 3 displays the evolution of pH and $[Fe^{3+}]/[Fe^{2+}]$ ratios during the incubation using water from different sampling stations. After four weeks of incubation only water samples from stations M01 and M15 were able to promote the precipitation of jarosite. M01 corresponds to the origin of the river (Izq–Izq in [35] and R1 [36]) and M15 corresponds to a channel existing in an abandoned mine site in which *P. lilacinum* M15001 was first isolated. No precipitation of jarosite could be observed for the media prepared with water from M07 (Richi in [37]), M11 (RT5 in [36]), M13 (3.2 in [35] and R5 in [36]) or M19 (Berrocal in [35] and RT10 in [36]) stations. In all cases the fungi were able to grow (Fig. 1). This result clearly shows that fungal development is not sufficient to promote the precipitation of jarosite. The ionic conditions existing in each media must be critical, although we cannot rule out the possible effect produced by these conditions on the structural components of the cell wall required for the specific nucleation of the mineral. The comparison of the physico-chemical conditions of the water samples indicate that the redox potential does not seem to be critical, because promoting and no promoting jarosite formation water samples exhibit similar Eh values. Some differences could be detected in the conductivity values, exhibiting the promoting water samples, M01 and M15, intermediate values when compared to the rest.

It is interesting to note that the ionic conditions that promote the specific generation of jarosite have quite different $[Fe^{3+}]/[Fe^{2+}]$ ratios. In M01 most of the iron was oxidized ($Fe^{3+}/Fe^{2+} = 4.61$), while in M15 most of the iron was reduced ($Fe^{3+}/Fe^{2+} = 0.27$). In the case of M01, the formation of jarosite could be explained as a consequence of the ionic conditions of the media, in which jarosite is supersaturated. In fact, control experiments with culture media without fungal inoculation, precipitated jarosite. In this case, globular nanoforms can be also seen associated to the fungal hyphae, probably acting as nucleation agents for a more efficient precipitation of jarosite (Fig. 1). This is not the case for M15, in which the ionic conditions predict the precipitation of other supersaturated minerals, like goethite [27]. It is clear from

Table 1
Generation of jarosite by different fungal isolates from the Río Tinto basin.

Phylum	Class	Order	Genus	Species/isolate	Jarosite formation
Ascomycota	Sordariomycetes	Hypocreales	*Purpureocillium*	*lilacinum* M15001	++
Ascomycota	Sordariomycetes	Hypocreales	*Purpureocillium*	*lilacinum* M01003	++
Ascomycota	Sordariomycetes	Hypocreales	*Purpureocillium*	*lilacinum* DSM846^T	++
Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma* sp.	sp. M01037s	–
Ascomycota	Sordariomycetes	Hypocreales	*Verticillium*	*leptobactum* M15005	–
Ascomycota	Sordariomycetes	Sordariales	*Chaetomium*	sp. M15023s	–
Ascomycota	Dothideomycetes	Capnodiales	–	M15027s	–
Ascomycota	Dothideomycetes	Capnodiales	–	M15021s	–
Ascomycota	Dothideomycetes	Capnodiales	*Bispora*	sp. M01004	–
Ascomycota	Dothideomycetes	Capnodiales	*Teratosphaeria*	sp. M02009	–
Ascomycota	Eurotiomycetes	Aspergillaceae	*Penicillium*	*dalae* M04004	–
Ascomycota	Eurotiomycetes	Aspergillaceae	*Penicillium*	*simplicissimum* M09005	–
Ascomycota	Eurotiomycetes	Aspergillaceae	*Penicillium*	*glabrum* M15024s	–
Ascomycota	Eurotiomycetes	Aspergillaceae	*Penicillium*	*griseolum* M15013s	–
Ascomycota	Eurotiomycetes	Aspergillaceae	*Penicillium*	*ochrochlon* M13005	–
Ascomycota	Eurotiomycetes	Aspergillaceae	*Aspergillus*	sp. M11005	–
Ascomycetes	Eurotiomycetes	Tricocomaceae	*Talaromyces*	sp. M02039	–
Basidiomycota	Agaricomycetes	Agaricales	*Pholiota*	sp. M15016	–
Zygomycota	Zygomycetes	Mortierelales	*Mortierella*	sp. M02002	–

Table 2
Physico–chemical characteristics of different Rio Tinto sampling stations. Eh corresponds to redox potential measured in mV; Cond; conductivity in mS cm^{-1}; T: temperature in °C; ionic concentration are given in mg L^{-1} and jar to the formation of jarosite in the media prepared with water from these sampling sites.

Station	pH	T	Eh	Cond.	O_2	K	$[Fe^{2+}]$	$[Fe^{3+}]$	$[SO_4^{2-}]$	[Cu]	[As]	[Co]	Jar
M01	2.0	25.6	505	24.4	4.6	3.3	2169	10,037	9679	14.9	12.0	20.4	++
M07	1.8	21.0	450	49.8	3.1	15.5	11,140	1089	10,756	82.0	31.1	14.8	–
M11	2.2	24.1	497	17.3	5.7	6.3	577	125	1007	8.0	0.7	1.0	–
M13	2.5	25.5	513	4.6	6.7	1.9	32	135	364	13.6	0.8	1.2	–
M15	2.4	20.9	397	16.8	2.8	1.5	4032	1112	5903	480.7	1.4	4.8	++
M19	2.6	26.3	643	5.9	8.7	1.6	93	801	1391	101.9	1.7	0.7	–

these results that the mechanism operating in M15 is quite different than the one precipitating jarosite for M01. Interestingly, M11 has a $[Fe^{3+}]/[Fe^{2+}]$ ratio similar to M15, but in this case the fungal growth does not promote the formation of jarosite (Fig. 1 and Tables 2 and 3). This result suggests that the concentration of Fe^{3+} and SO_4^{2-} must be also critical for generating the nucleation effect. Although the $[Fe^{3+}]/[Fe^{2+}]$ ratios are high enough in M13 and M19 cultures (Table 3), precipitation in the control experiments without fungal inoculation was not observed, which suggest that the Fe^{3+} concentration is not high enough to reach the mineral supersaturation obtained in M01. In both cases an effective fungal growth has not been able to nucleate jarosite formation (Fig. 1).

In our previous work, in which specific hydronium-jarosite precipitation culturing *P. lilacinum* was observed, it was concluded that the chemistry of the culture media indicated overall disequilibrium between the Eh, $O_{2,aq}$ and Fe^{3+}/Fe^{2+} ratio, consistent with its turbulent flow and precipitation dissolution processes [27]. The measured Eh values suggested negative saturation indexes (SI) for jarosite at all the expected phases in the system. The SI relates the theoretical log K_{diss} of a solid phase with the real activity product of the solution. Mineral phases with positive or close to cero SI values can precipitate from solution, while minerals having SI < 0 are undersaturated and cannot precipitate. At constant pH and temperature only a change in the concentration of K^+, SO_4^{2-} and F^{3+}can supersaturate jarosite, with the change of the Fe^{3+} concentration being the most effective mechanism. Thus, our model proposed that an increase in the Fe^{3+}/Fe^{2+} ratio in the immediate environment of the cells could rapidly supersaturate and precipitate jarosite. Numerical modeling using the GWB code suggested than an increase in the $\sum Fe^{3+}/\sum Fe^{2+}$ aqueous ratio could have a strong influence in the SI values [27]. The results presented in this work using different ionic conditions agree with this model. Only M15 is able to promote specific precipitation of hydronium-jarosite in undersaturated conditions. M01 can produce it but at supersaturated conditions. Other conditions (Tables 2 and 3) are not able to give the SI values required for jarosite precipitation. None of these conditions had any effect on the fungal growth, as shown in Fig 1. Thus the only presence of nucleation sites is not sufficient to promote mineral precipitation, adequate ionic conditions are also required.

Our results clearly show that the ratio of the redox pairs and the Fe^{3+} concentration is important to achieve a specific biomineralization of jarosite. The amount of nucleation sites also seems to be critical. However the detailed mechanism that this fungus specifically uses to saturate hydronium-jarosite and not goethite or hematite, the minerals expected to precipitate due to an increase in the Eh, is unknown.

Preliminary experiments showed that non-viable cells were able to induce the formation of hydronium-jarosite although with much less efficiency than viable cells [27]. To further evaluate the influence of carbon and energy source in the formation of jarosite, *P. lilacinum* was grown in the absence of the organic components used to amend the M15 media. The absence of glucose or the three added organic components

Table 3
Evolution of pH and $[Fe^{3+}]/[Fe^{2+}]$ ratios during the growth of *P. lilacinum* in different conditions. pH_i, initial pH, pH_f, final pH, $[Fe^{3+}]/[Fe^{2+}]_i$ initial ratio of oxidized and reduced iron, $[Fe^{3+}]/[Fe^{2+}]_f$ final ratio of oxidized and reduced iron.

Strain	Sampling station											
	M01				M07				M11			
	pH_i	pH_f	$[Fe^{3+}]/[Fe^{2+}]_i$	$[Fe^{3+}]/[Fe^{2+}]_f$	pH_i	pH_f	$[Fe^{3+}]/[Fe^{2+}]_i$	$[Fe^{3+}]/[Fe^{2+}]_f$	pH_i	pH_f	$[Fe^{3+}]/[Fe^{2+}]_i$	$[Fe^{3+}]/[Fe^{2+}]_f$
P. lilacinum M15001	2.0	1.5	4.61	2.7	1.8	1.2	0.09	0.09	2.2	1.4	0.21	0.21
P. lilacinum DSM 846^T	2.0	1.7	4.61	2.8	1.8	1.3	0.09	0.09	2.2	1.5	0.21	0.21
P. lilacinum M15001*	2.0	2.0	4.61	3.1	1.8	1.8	0.09	0.09	2.2	2.2	0.21	0.21
Non-inoculated control	2.0	2.0	4.61	3.2	1.8	1.8	0.09	0.09	2.2	2.2	0.21	0.21
Strain	M13				M15				M19			
	pH_i	pH_f	$[Fe^{3+}]/[Fe^{2+}]_i$	$[Fe^{3+}]/[Fe^{2+}]_f$	pH_i	pH_f	$[Fe^{3+}]/[Fe^{2+}]_i$	$[Fe^{3+}]/[Fe^{2+}]_f$	pH_i	pH_f	$[Fe^{3+}]/[Fe^{2+}]_i$	$[Fe^{3+}]/[Fe^{2+}]_f$
P. lilacinum M15001	2.5	2.0	4.2	4.2	2.5	2.0	0.27	0.19	2.6	2.0	8.6	8.6
P. lilacinum DSM 846^T	2.5	2.0	4.2	4.2	2.5	2.2	0.27	0.21	2.6	2.2	8.6	8.6
P. lilacinum M15001*	2.5	2.5	4.2	4.2	2.5	2.5	0.27	0.24	2.6	2.6	8.6	8.6
Non-inoculated control	2.5	2.5	4.2	4.2	2.5	2.5	0.27	0.27	2.6	2.6	8.6	8.6

T: type strain, *non-viable cells.

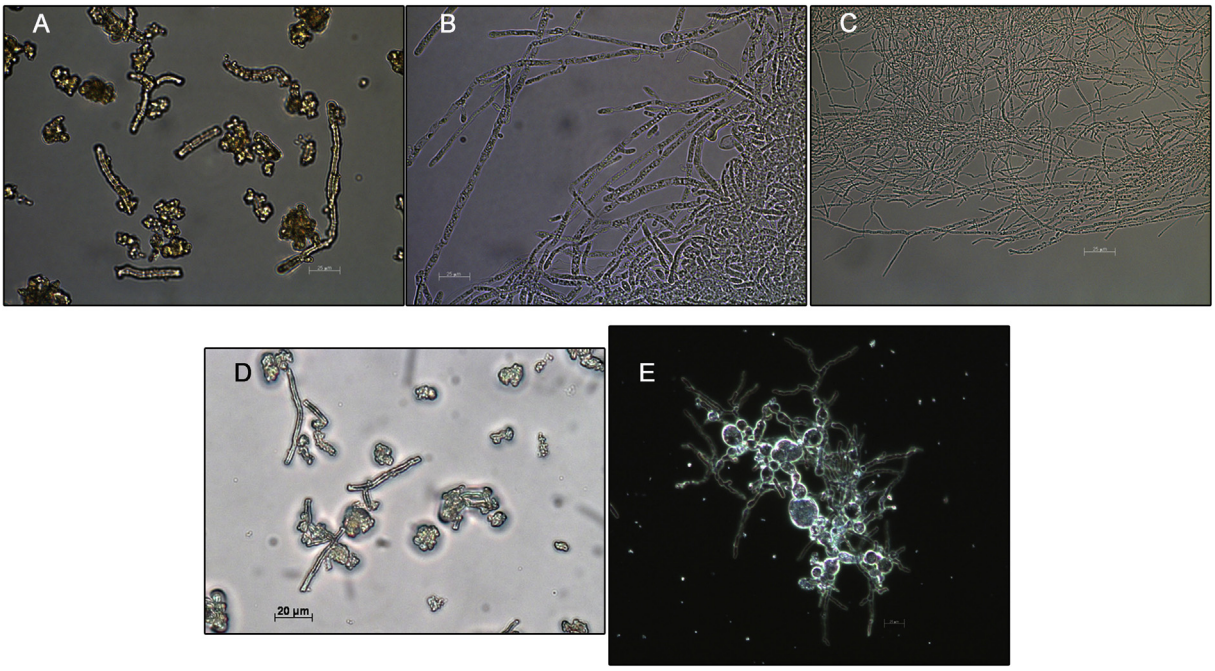

Fig. 1. Optical micrographs of *P. lilacinum* M15001 grown in cultures prepared with water from different Río Tinto sampling stations. (A) Fungal biomass grown in M01 culture medium. The presence of mineral phases which correspond to jarosite can be seen; globular structures associated with the fungal cell walls indicate that the biomineralization process is taking place on them. (B) *P. lilacinum* M15001 grown in M07 culture medium. No biomineralization of jarosite has been detected under this condition. (C) *P. lilacinum* M15001 grown in M13 culture medium. As in B there is no biomeneralization of jarosite. (D) *P. lilacinum* M15001 grown in M15 conditions with the presence of jarosite biomineralized hyphae. (E) *P. lilacinum* M15001 grown with M11 water. No biomineralization of jarosite has been detected in this condition.

(glucose, malt and yeast extracts) did not allow an efficient fungal growth and the formation of jarosite. A critical amount of nucleation sites must be also an important factor for the specific precipitation of jarosite.

One of the goals of this work was to ascertain the possible role of *P. lilacinum* in the generation of jarosite in the Tinto basin, a mineral recurrently present along its course (Table 4). As shown in Table 1, *P. lilacinum* has been isolated so far in stations M01 and M15. In both stations hydronium jarosite has been identified (Table 4). As discussed above, in M01 could be the product of supersatutation conditions, although as shown in Fig. 1 the presence of the fungi could increase the efficiency of the mineral precipitation. The lack of jarosite precipitation in M07 fits quite well with the existing undersaturated conditions and the absence of the fungi, although the presence of jarosite in other stations, especially in M19, in which *P. lilacinum* has not been isolated, does not fit with the model. It is clear from these results that other mineral precipitation effectors have to be considered. We have to keep in mind that we are dealing with a river, and only a thorough study directed to answer this question might give some clues on the subject. In any case, our data clearly show that the nucleation effect of *P. lilacinum* has to be considered in the generation of this peculiar mineral in the Tinto basin and in any other system in which has been identified [38–40].

Table 4
Identification of jarosite in different Río Tinto locations according to Fernández-Remolar et al. (2005). na: not analyzed.

Station	Jarosite identification
M01	+
M07	–
M11	+
M13	na
M15	+
M19	+

Considering that the precipitation of jarosite is removing the effective metal sulfides bioleaching agent, ferric iron, from solution; that jarosite is an efficient metal sequester and its ability to passivate chalcopyrite protecting the mineral from further bioleaching attack [41–44], we think that it should be of interest to control the development of *P. lilacinum* in bioleaching operations. From our results it is clear the effect that the presence of *P. lilacinum* nucleation sites can have in the generation of jarosite. Knowing the effect of low pH in preventing the formation of jarosite, it is clear that the control of this effector is the best way to avoid its generation. This can be easily achieved in stirred tank operations. But considering that these methodologies are of use only in economically productive processes and heap leaching is used in most of the bioleaching operations, it will be very convenient to control the presence and growth of this fungi, especially when solvent extraction procedures can increase the organic compounds in the bioleaching solutions, favoring the growth of heterotrophic microorganisms, like fungi.

Concerning its possible use, enhancement of jarosite precipitation by the presence of *P. lilacinum* could be of interest in

the zinc industry in which jarosite is used to remove iron in the processing circuit. Normally jarosite is added to the solution to accelerate the precipitation reaction [45]. The introduction of a nucleation agent like *P. lilacinum* could improve the efficiency of the process. Following the same idea, *P. lilacinum* could be of use to facilitate removal of sulfate and ferric iron from any metallurgical process that might require it.

4. Conclusions

The specificity of *P. lilacinum* to precipitate hydronium-jarosite in undersaturated conditions has been proved using an extensive number of acidophilic fungi isolated from the Río Tinto basin, covering a wide range of fungal phyla. Only this fungus from the order Hypocreales has this ability. The effect of the ionic conditions in the formation of jarosite has been tested using water collected from different sampling sites. From the results can be concluded that the concentration of ferric iron, the ratio between Fe^{3+}/Fe^{2+} and the presence and amount of nucleation sites are critical factors for the precipitation of jarosite, although the presence of nucleation sites by themselves is not sufficient to promote jarosite precipitation. There is a good correlation between the sampling sites along the river in which *P. lilacinum* has been isolated and hydronium-jarosite identified. The control of the growth of *P. lilacinum* is advisable in processes in which the pH is not easy to control, like heap leaching operations, to prevent ferric iron precipitation and metal sequestration.

Conflict of interest

None.

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