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Testing the Capacity of *Staphylococcus equorum* for Calcium and Copper Removal through MICP Process

Sebastián Sepúlveda [1], Carla Duarte-Nass [1,2,3], Mariella Rivas [4], Laura Azócar [5], Andrés Ramírez [6], Javiera Toledo-Alarcón [7], Leopoldo Gutiérrez [6], David Jeison [7] and Álvaro Torres-Aravena [7,*]

1 Departamento de Ingeniería Química, Universidad de La Frontera, Temuco 4811230, Chile; s.sepulveda04@ufromail.cl (S.S.); carla.duarte@ufrontera.cl (C.D.-N.)
2 Núcleo Científico y Tecnológico de Biorecursos (BIOREN), Universidad de La Frontera, Temuco 4811230, Chile
3 Centro de Recursos Hídricos Para La Agricultura y La Minería (CRHIAM), Concepción 4030000, Chile
4 Laboratorio de Biotecnología Ambiental Aplicada BIOAL, Dpto. de Biotecnología, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, Antofagasta 1270300, Chile; mariella.rivas@uantof.cl
5 Departamento de Química Ambiental, Facultad de Ciencias, Universidad Católica de La Santísima Concepción, Concepción 4090541, Chile; lazocar@ucsc.cl
6 Departamento de Ingeniería Metalúrgica, Facultad de Ingeniería, Universidad de Concepción, Concepción 4030000, Chile; aramirezm@udec.cl (A.R.); lgutierrezb@udec.cl (L.G.)
7 Escuela de Ingeniería Bioquímica, Facultad de Ingeniería, Pontificia Universidad Católica de Valparaíso, Valparaíso 2362803, Chile; javiera.toledo@pucv.cl (J.T.-A.); david.jeison@pucv.cl (D.J.)
* Correspondence: alvaro.torres@pucv.cl

Abstract: This research focused on the evaluation of the potential use of a soil-isolated bacteria, identified as *Staphylococcus equorum*, for microbial-induced calcite precipitation (MICP) and copper removal. Isolated bacteria were characterized considering growth rate, urease activity, calcium carbonate precipitation, copper tolerance as minimum inhibitory concentration (MIC) and copper precipitation. Results were compared with *Sporosarcina pasteurii*, which is considered a model bacteria strain for MICP processes. The results indicated that the *S. equorum* strain had lower urease activity, calcium removal capacity and copper tolerance than the *S. pasteurii* strain. However, the culture conditions tested in this study did not consider the halophilic feature of the *S. equorum*, which could make it a promising bacterial strain to be applied in process water from mining operations when seawater is used as process water. On the other hand, copper removal was insufficient when applying any of the bacteria strains evaluated, most likely due to the formation of a copper–ammonia complex. Thus, the implementation of *S. equorum* for copper removal needs to be further studied, considering the optimization of culture conditions, which may promote better performance when considering calcium, copper or other metals precipitation.

Keywords: mining process water; microbial-induced calcite precipitation (MICP); calcium; copper removal

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1. Introduction

Water scarcity is nowadays a serious problem for many societies, which has trigged a variety of efforts towards smart and sustainable management, treatment and reuse of this vital element. Industry is called to play an active role in the framework of such efforts, especially when water is relevant for the process involved.

Mining is a water-intensive industry because of water consumption associated with extraction, processing and disposal of minerals. Moreover, water use for mining is often hampered by the geographical location of the operations, which sometimes includes desert zones, as is the case of Chile, a worldwide leader in copper mining [1,2]. Additionally, the decreasing ore grade of many mining deposits demands a higher volume of process water [3]. Moreover, mining operations usually impact the environment because of the

disposal of tailings and wastewaters that sometimes contaminate the soil and nearby watercourses with heavy metals [4]. Thus, water recirculation and wastewater treatment are focuses of special interest for the mining industry when conceiving the future projection of this activity.

When it comes to addressing the issue of water scarcity in mining, several strategies have been developed and implemented, such as the use of new water resources (seawater or saline water) [5] and water recovery from thickeners and tailings dams. However, these alternatives create new challenges. One of them is the presence of dissolved ions during the flotation process, especially when seawater is used as process water. When water recirculation or seawater use is considered, plant performance may be affected due to the increase in dissolved ions and ionic strength [6,7]. In fact, there are studies that show how ions from seawater, especially calcium and magnesium, can affect flotation of copper ores [8]. Moreover, during the flotation of sulphide ores, slaked lime ($Ca(OH)_2$) is applied intensively to control alkalinity, to depress pyrite and to improve water recovery from tailings [9], which generates high calcium concentration in water loops. Thus, there is a real necessity to develop technologies capable of reducing calcium concentration, improving flotation performance. In addition, recovery technologies of valuable metals from the process water, before its discharge with tailings, could reduce the risk of contamination of the environment. In this context, the biotechnological processes represent a sustainable option, due to their mild operational conditions and low energetic requirements.

Microbial-induced calcite precipitation (MICP) has been proposed as a potential process for calcium and heavy metal removal from soils and (waste)waters [10,11]. MICP is based on the capacity of many non-pathogenic bacteria to hydrolyze urea, releasing carbonate and ammonium [12]. The released ammonium, a product of urea hydrolysis, increases the pH, promoting the precipitation of calcite when calcium ions are present [13]. The potential application of MICP to copper removal is sustained on the fact that divalent ions, such as copper itself, can bind to carbonate ions, precipitating as carbonates [14,15]. Thus, MICP could be applied in waters when the recovery of calcium and copper is of interest, such as recirculating water and wastewaters from mining operations, contributing to reduce lime consumption, and preventing long-term copper pollution of the environment.

Application of the MICP process for calcium recovery would release ammonium into mineral processing. Although the potential effect of this ammonium is not known in detail, recent publications suggest that ammonium can improve the selectivity of arsenopyrite and chalcopyrite. In addition, ammonium could promote the formation of cuprous xanthate and dioxanthogen [16], which improve the flotation of copper and molybdenum sulphides, possibly generating a double benefit when MICP is applied.

Factors governing MICP are pH, urea, calcium concentrations and the type of bacteria used [13,17]. Considering the latter factor, some authors have reported successful calcium and heavy metal removals when using bacteria strains isolated from contaminated soils with heavy metals [18–21]. Reported removal efficiencies can be as high as 90–97% [22–24]. A large fraction of soil bacteria can hydrolyze urea through urease enzyme [25]. Thus, the isolation of soil bacteria could be a useful approach when searching for strains with MICP capacity and heavy metals tolerance.

Thus, this research was focused on the isolation, identification and characterization of strains with MICP potential for calcium and copper removal from synthetic process water enriched with these metals.

2. Materials and Methods

2.1. Bacterial Isolation and Selection

Soil samples with an abundance of heavy metals were collected using sterile tubes from a coastal zone, close to Sotramin tailings deposit, in the city of Taltal, Chile (23°42′11.35″ S 70°25′27.54″ O). An amount of 1 g of soil was suspended in 50 mL of nutrient broth (NB) composed by beef extract 1 g/L, peptone 5 g/L, yeast extract 2 g/L and NaCl 5 g/L. A series of dilutions were carried out, and those showing bacterial growth were subsequently

grown in plates at 30 °C, using the medium already mentioned. The colony-forming units (CFU) were subcultured to identify the presence of microorganisms with urease activity. To identify urease activity, strains were grown in Eppendorf tubes at 37 °C overnight with modified Christensen's urea agar (NaCl 5 g/L, peptone 1 g/L, glucose 1 g/L, KH_2PO_4 2 g/L, phenol red 0.012 g/L, agar 15 g/L and urea 20 g/L, pH 6.5). This method is based on a qualitative detection of urease activity through the change of color of the medium, from yellow to pink. Three bacterial cultures were identified as urease-positive with Christensen's method, which were chosen for characterization and identification (named A6, A11 and A22). As will be presented later, all three isolated strains corresponded to *S. equorum*. The strain *S. pasteurii* NCIMB 8841 was used for comparison purposes, as model bacteria and positive control, due to its urease activity and calcite precipitation capacity.

2.2. Bacterial Identification by 16S rRNA Gene, Phylogenetic Analysis and Nucleotide Sequence Accesion Numbers

To identify isolates, strains were grown overnight and centrifuged at 5000× *g* for 5 min. Total DNA was extracted through DNeasy Power Soil kit (Qiagen Cat. No. 12888-100, Qiagen, Hilden, Germany) following the manufacturer's instructions. Using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′TACGGYTACCTTGTTACGACTT-3′), the 16S rDNA gene was amplified by PCR [26] and then sequenced at Macrogen Inc. (Seoul, South Korea). The software CLC Main Workbench version 6.7.1 (Qiagen, Hilden, Germany) was used to edit sequences. Sequence assembling was performed using version Chromas Pro 1.6 (2012, Technelysium, Cordelia, South Brisbane, Australia) and then analyzed by BLASTN database (http://www.ncbi.nlm.nih.gov, accessed on: 22 July 2021), using the non-redundant GenBank nucleotide collection. Evolutionary analyses were conducted in MEGA X software [27] using Maximum Likelihood, with a bootstrap of 1000. The model used was General Time Reversible. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The 16S rRNA gene sequences obtained from tailings soil strains were uploaded to GenBank with the following accession numbers: MZ603727 (strain A6), MZ603729 (strain A11) and MZ603730 (strain A22).

2.3. Bacterial and Mineral Characterization

Pure cultures were cultivated in 100 mL of NB medium and 2% urea (20 g/L), in 250 mL flasks. Growing conditions were pH 7, 30 °C and shaking at 130 rpm. The cellular growth was determined during growth assays through tracking the optical density at a wavelength of 600 nm (OD_{600}). Biomass concentration was computed through conversion of OD_{600} to suspended solids (SS) using a calibration curve. A specific calibration curve was recorded for every strain studied in this research, expressing biomass concentration as suspended solids. Assays were performed in triplicate.

Calcite production was determined using a precipitation media, composed of beef extract 3 g/L, urea 20 g/L, $CaCl_2 \times 2H_2O$ 28.5 g/L, $NaHCO_3$ 2.12 g/L and NH_4Cl 10 g/L. An amount of 142.5 mL of the precipitation media was inoculated with 7.5 mL of liquid bacterial culture in 250 mL flasks. Strains were incubated for 5 days at 30 °C and 130 rpm. Precipitates were separated by centrifugation at 6000 rpm for 10 min and later dried at 37 °C. The produced $CaCO_3$ was measured by reverse titration. Samples of the precipitates were collected, and its composition was analyzed through scanning electronic microscopy coupled with energy-dispersive X-ray (SEM-EDX, SU 3500, HITACHI, Tokyo, Japan). The amount of biomass embedded in the precipitates was determined as the difference between the total mass of precipitates (weighted after centrifugation and drying) and the $CaCO_3$ (measured through reverse titration).

Urease activity for selected strains was determined through the phenol-hypochlorite method, according to [22]. A sample of pre-filtrated culture media (250 µL) was added to a mixture containing 1 mL of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 mL of urea (0.1 M). The mixture was incubated at 37 °C for 5 min. Then, 1 mL of both phenol nitroprusside and alkaline hypochlorite were added, and the mixture was incubated at 37 °C for 25 min. Optical density was measured using a spectrophotometer at a wavelength

of 626 nm. One unit of urease was defined as the amount of enzyme hydrolyzing 1 µmol urea per min.

2.4. Copper Tolerance and Copper Removal Assay

Copper tolerance was determined to be in the range of 0–4 mM. Different copper concentrations were achieved by the addition of $CuCl_2 \times 2H_2O$. Growth media were the same as described in Section 2.1. Values for the minimal inhibitory concentration (MIC) were computed according to [28]. MIC is defined, for the purpose of this study, as the copper concentration providing a 70% reduction in growth.

Copper removal was evaluated cultivating *S. pasteurii* and *S. equorum* in the precipitation medium already described, supplemented with 1.46 mM of Cu^{+2}. This copper concentration was selected based on the results of copper tolerance assays, considering the higher copper concentration not providing complete inhibition. The assay was carried out for 7 days, at 30 °C and 130 rpm. Once the assay was finished, the culture was centrifugated at 6000 rpm for 10 min. Supernatant was analyzed for copper concentration, using atomic absorption spectroscopy (SensAA Dual model—GBC Scientific Equipment, Melbourne, Australia). Removal of copper was computed considering initial and final copper content of the liquid phase. Assays were performed in triplicate.

2.5. SEM-EDX

Variable pressure scanning electron microscopy (SEM), with transmission module STEM SU-3500 (Hitachi, Tokyo, Japan) and a QUANTAX 100-energy-dispersive X-ray (EDX) detector (Bruker, Billerica, MA, USA), was used to semi-quantitatively analyze the compositions of the precipitates.

3. Results

3.1. Isolation and Identification of Bacterial Culture with Ureolytic Activity

A total of 22 possible strains (named A1–A22) were isolated from the soil samples. All isolates were tested for the presence of urease activity. Three isolates were selected based on the time required for color turn when using Christensen's media, considering that a lower time means a higher activity. Thus, three isolates with high ureolytic activity were identified phylogenetically based on 16S rRNA gene sequences through BLASTN (Table 1). The results indicated that all three selected isolates (A6, A11 and A22) correspond to *Staphylococcus equorum*, with a 99% similarity (99–100% coverage) (Table 1). A phylogenetic tree was generated based on the 16S rRNA. The relatedness of the sequences is shown in Figure 1. *S. equorum* has already been reported as a urease-positive bacteria [29]. Isolated A6, A11 and A22 were cultivated in liquid medium, and growth rates were individually determined. A11 isolated showed the higher growth rate and calcite production (data not shown), so it was selected for the following experiments. This isolate, from now on, will be simply referred to as *S. equorum*.

Table 1. Phylogenetic identification of ureolytic bacterial strains isolated from tailings.

Bacterial Strain	Closest Species in BLASTN	E Value	Coverage (%)	Identity (%)	Homolog GenBank Accession Number
A6	*Staphylococcus equorum* strain PL453	0.0	100	99.52	MK015791.1
A11	*Staphylococcus equorum* strain PL453	0.0	99	99.32	MK015791.1
A22	*Staphylococcus equorum* strain PL453	0.0	99	99.73	MK015791.1

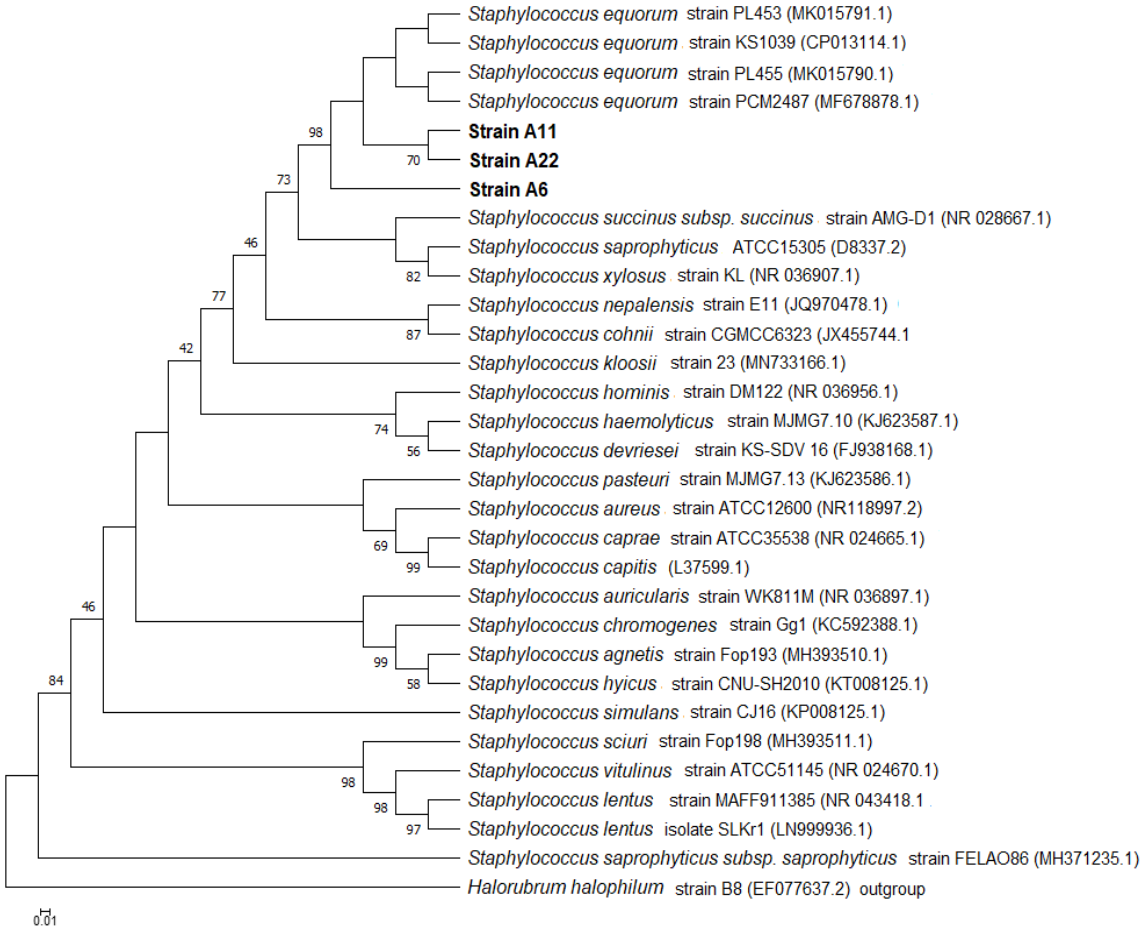

Figure 1. Molecular phylogenetic analysis by Maximum Likelihood method and General Time Reversible model [30]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [27]. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [27]. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances, estimated using the Maximum Composite Likelihood (MCL) approach. This analysis involved 31 nucleotide sequences. There were a total of 1670 positions in the final dataset. All positions containing gaps and missing data were eliminated. Thus, the strains A6, A11 and A22 are included in the analysis. The scale bar indicates the branch lengths measured in the number of substitutions per site.

Several *Staphylococcus* strains have been isolated from soil and sand in the context of MICP research. This is the case of *S. epidermidis* and *S. saprophyticus* [31,32]. However, to the knowledge of the authors, there is no published research using *S. equorum* for MICP applications.

3.2. Ureolytic Activity of S. equorum

3.2.1. Bacteria Growth

The results in Figure 2 show growth curves for *S. equorum* and *S. pasteurii* (used as model bacteria for MICP). Final biomass concentrations were close to 1 g/L and 2 g/L, respectively, after 24 h. Specific growth rate was computed, resulting in values of 0.24 and 0.58 h^{-1} for *S. equorum* and *S. pasteurii*, respectively.

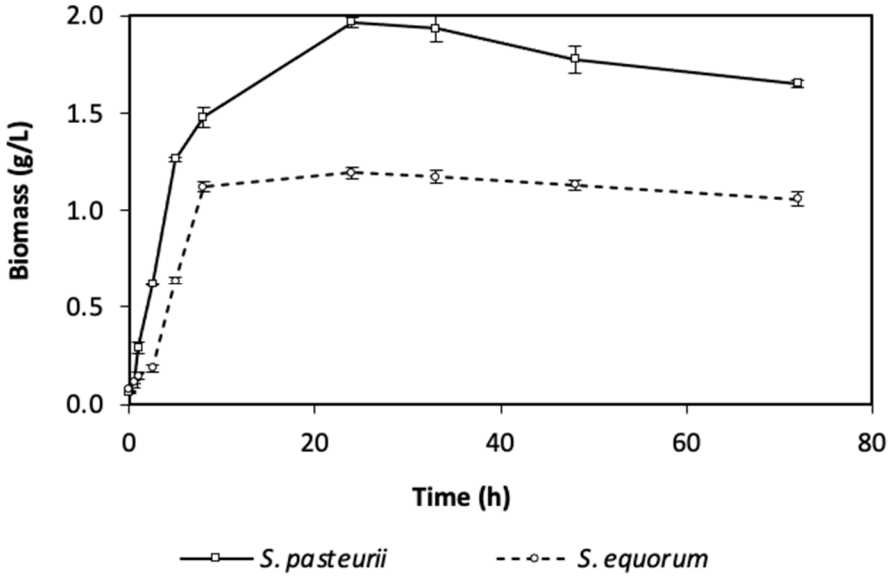

Figure 2. Growth curves for *S. pasteurii* and *S. equorum*. Bars indicate standard deviation.

Figure 3 shows enzymatic activity evolution during growth assays. In the case of *S. pasteurii*, a maximal activity of 10 U/mL was observed by the end of the assay (72 h). *S. equorum* also showed a urease activity that increased with time, until a value of 5 U/mL. Similar values of urease activity have been reported for isolated strains from contaminated soils, ranging from 2 to 20 U/mL [21,24]. However, other authors have reported higher levels of urease activity [20,22,33].

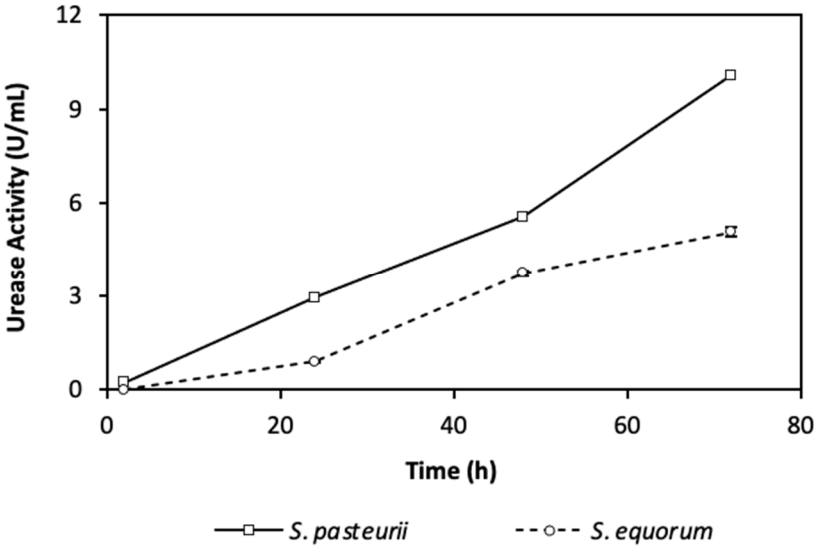

Figure 3. Urease activity of *S. pasteurii* and *S. equorum* during growth assays.

3.2.2. $CaCO_3$ Precipitation

Figure 4 shows the results of the $CaCO_3$ production assays. *S. pasteurii* and *S. equorum* induced $CaCO_3$ precipitation of 4.9 and 3.5 g/L. Researchers have reported a high urease activity and therefore $CaCO_3$ precipitation capacity for *S. pasteurii*, which is why this microorganism is normally considered a "model bacteria" for MICP [17,33,34]. Other studies have reported, for isolated strains from contaminated soils, similar values to the one registered for *S. equorum*, in the range between 5 and 17 mg/L $CaCO_3$ [18,19,21,35]. Nevertheless, this result could be improved by changing the culture conditions for *S.*

equorum, since it is reported that this bacteria strain can tolerate high salt concentration [36]. If the high salt tolerance of *S. equorum* is considered, this species could be a promising alternative for promoting calcite precipitation in waters from mining operations that consume seawater, where calcium and magnesium removal is needed.

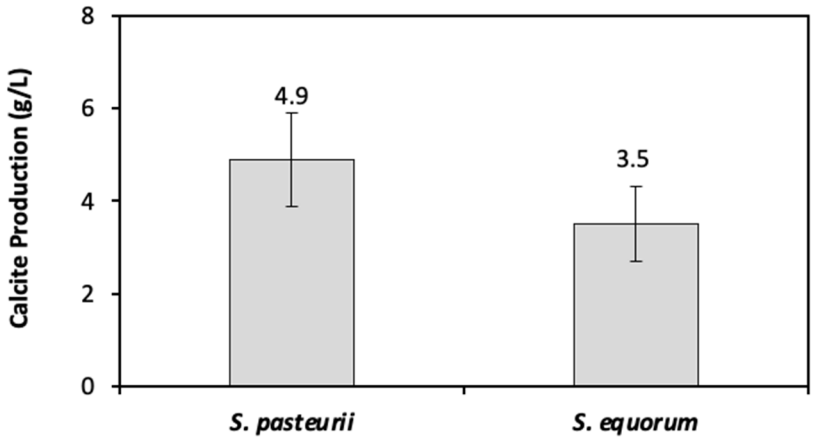

Figure 4. Calcite production by the end of precipitation assays for *S. pasteurii* and *S. equorum*. Bars indicate standard deviation.

Solids recovered from precipitation assays presented calcium compositions that were between 80% and 95% calcite. The rest is, most likely, biomass (bacteria) and/or solids remaining from the precipitation media. Differences in the non-calcite content of the recovered solids may be, at least partially, the result of different levels of biomass growth during the precipitation assay. During the precipitation assay of *S. pasteurii*, samples were taken to observe the development of calcite crystals. Figure 5 shows SEM images acquired at days 1, 3 and 5 of precipitation assays. By day 1 it was possible to identify bacillus shapes in the precipitates (red circles) that indicate the presence of bacteria on $CaCO_3$ crystals. Bacteria are expected to provide nucleation sites for $CaCO_3$ crystallization. As a result, they end up being embedded by the growing crystals [14,17,37]. By days 3 and 5, $CaCO_3$ formation continued, increasing the crystal radius until bacteria were embedded into the $CaCO_3$ structure.

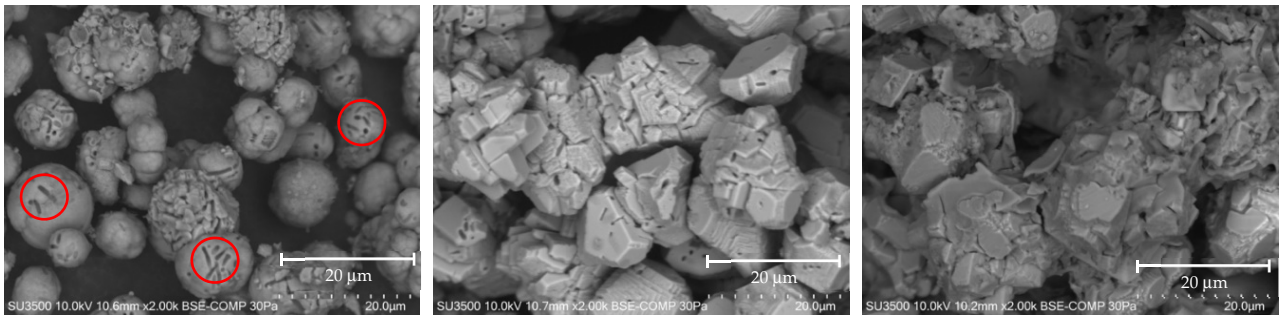

Figure 5. SEM images from solids recovered during $CaCO_3$ precipitation assays applying *S. pasteurii*. Day 1 (**Left**), day 3 (**Middle**) and day 5 (**Right**). Red circles in Figure (**Left**) show bacillus shapes indicating the presence of *S. pasteurii* in the precipitate.

To confirm the formation of $CaCO_3$ precipitates, the solids obtained by the action of *S. equorum* and *S. pasteurii* were analyzed through SEM-EDX. Table 2 shows the elemental composition of precipitates. The content of calcium was proportionally higher than the theorical one for calcium carbonate. Higher content of calcium could be explained by the interaction of this element with extracellular polymeric substances (EPS), produced by the bacteria metabolism as a response to a toxic environment [38]. Some studies have reported

that exopolysaccharides produced by microorganisms are responsible for biomineralization of several forms of calcium and other metal carbonates [39,40].

Table 2. Semi-quantitative elemental composition determined by SEM-EDX for precipitates obtained from calcite production assay and theorical composition of calcium carbonate.

Samples	Elemental Composition (%)			
	C	O	Ca	Other
S. pasteurii	7.95	29.72	51.61	10.72
S. equorum	9.65	34.94	43.63	11.78
$CaCO_3$	12.00	48.00	40.00	-

3.2.3. Copper Tolerance

Copper tolerances of *S. equorum* and *S. pasteurii* were evaluated by determining biomass growth at copper concentration between 0 and 4 mM. The results, shown in Figure 6, indicate that MICs for *S. pasteurii* and *S. equorum* were around 1.5 and 0.8 mM, respectively, when biomass growth after 72 h was considered. Different researchers have reported MIC values for copper containing media in the range 0.2–0.5 mM for *S. pasteurii* [28], 0.2–0.5 mM for *B. subtilis* [41], 0.5–0.8 mM for *Streptomyces* sp. [42] and 3.9–4.7 mM for *Sphingomonas paucimobilis* [43]. On the other hand, some authors have reported high metal tolerance for bacteria strains isolated from contaminated soils, showing values up to 23 mM [44]. According to the results of this study, *S. equorum* has a lower copper tolerance than *S. pasteurii*. However, an optimization on the culture medium for *S. equorum* could improve the copper tolerance, as was also commented before for calcium removal. Additionally, heavy metal's toxic effect on biomass growth could be overcome by a two-step process, where a first operation would produce the biomass, in absence of the toxic elements, with a second operation dedicated to the precipitation of calcium and/or heavy metals, as proposed by [10,45].

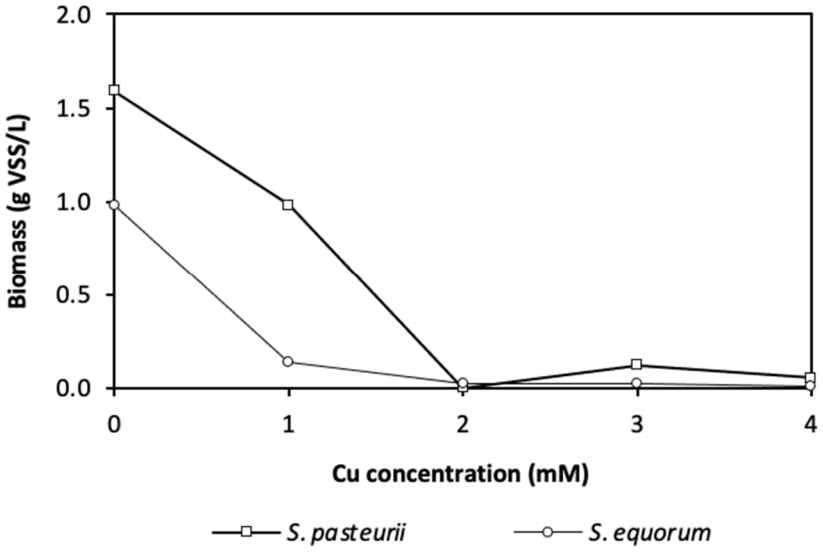

Figure 6. Observed biomass growth of *S. pasteurii* and *S. equorum* observed after 72 h cultivation, at different copper concentrations.

3.2.4. Copper Removal

To evaluate the potential capacity of the isolated *S. equorum* to remove copper from process water from mining operations, copper precipitation assays were carried out. An assay including *S. pasteurii* was included for comparison purposes. The results indicated similar copper removal from the liquid phase, 11% with *S. pasteurii* and 10% with *S. equorum*. From a statistical point of view, there were no significant differences between copper

removal for either species. These values are insufficient to support a potential application in a mining operation, where the removal of metals should be significant to justify the investment. Reports have shown low copper removals at copper concentrations over 0.5 mM and, on the contrary, high copper removals at lower copper concentrations [28,46]. The observed behavior could be related to copper-driven inhibition. Nevertheless, a poor copper carbonate precipitation could also be the result of the formation of a copper–ammonia complex, as was reported by [45] when working at similar conditions, using *S. pasteurii*. Copper–ammonia complexes are soluble and stable. Their formation could prevent copper carbonate bonding and subsequent precipitation [47]. This issue would require pH control to induce the formation of copper carbonates, as indicated by [45].

4. Discussion: Potential Application in Mining Operations

Despite the absence of published research dealing with the application of *S. equorum* in MICP-based processes for mining operations, other *Staphylococcus* strains have been proposed as bioremediation agents for heavy metals through calcium carbonate precipitation. The authors of [31] reported lead co-precipitation with calcium carbonate applying *S. epidermidis* as a tool for wastewater treatment, achieving 86% of lead removal. During that study, reported urease activity was on the order of magnitude of the one determined in this study for *S. equorum*. On the other hand, biosorption has been described as another technology for heavy metals bioremediation. In this sense, *S. xylosus*, *S. epidermidis* and *S. saprophyticus* have been reported as bio-sorbents for cadmium, chromium and lead removal, respectively [48–50].

Calcium recovery from process water in mining operations through MICP applying *S. equorum* has potential and deserves to be explored, considering that the calcium removal measured in this study was not carried out in optimal culture conditions. Kim et al. [32] reported that *S. saprophyticus* isolated from sand samples precipitated 5 times more calcium carbonate than *S. pasteurii*, under optimal culture conditions. Even though application of *S. equorum* has not been applied in environmental biotechnology studies, this bacterium has been well described in food-related research of cheese, ham and high-salt-fermented seafood [51–53]. The halophilic characteristic of *S. equorum* could make it a promising bacteria strain to be applied to selected metal removal processes in waters from mining operations with a high content of sodium, calcium and/or magnesium. Therefore, it is imperative that forward research aims on the optimization of *S. equorum* culture conditions, which may promote calcium or other metals precipitation from recirculating water in mining operations.

5. Conclusions

S. equorum was successfully isolated from potentially heavy metal contaminated soil. Its performance was evaluated, in comparison with *S. pasteurii*, as MICP model bacteria. The results indicated that *S. equorum* had lower urease activity, calcium removal and copper tolerance than *S. pasteurii*. However, the culture conditions tested in this study did not consider the halophilic feature of *S. equorum*. This characteristic may present *S. equorum* as a promising bacterial strain to be applied in process water from mining operations, where a high content of ions is present as a result of, for example, the use of seawater. The optimization of culture conditions for calcium precipitation will be crucial for the future implementation of this technology. On the other hand, copper precipitation would require additional operations to tackle chemical interactions between copper and ammonia generated by *S. equorum* metabolism. Thus, the implementation of *S. equorum* for copper removal from process water in mining operations demands further research.

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