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Bioleaching of electronic waste using bacteria isolated from the marine sponge *Hymeniacidon heliophila* (Porifera)

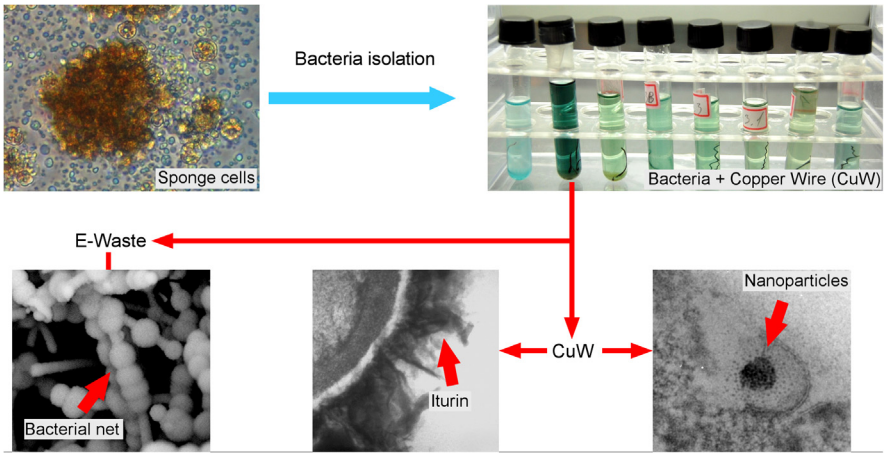
Enrique E. Rozas[a,*], Maria A. Mendes[a], Claudio A.O. Nascimento[a], Denise C.R. Espinosa[b], Renato Oliveira[c], Guilherme Oliveira[c], Marcio R. Custodio[d]

[a] *LSCP, Chemical Engineering Department, University of São Paulo (USP), Brazil*
[b] *LAREX, Chemical Engineering Department, University of São Paulo (USP), Brazil*
[c] *Vale Institute of Technology, Belém, PA, Brazil*
[d] *Departamento de Fisiologia Geral, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil*

HIGHLIGHTS

- A bioleaching mechanism of e-waste by bacteria-sponge associated starting in neutral pH is proposed.
- Bacteria secreted substances that binding to the leached copper and metallic nanoparticles were produced inside the cells.
- The substances linked to copper were identified as Iturin lipopeptide.
- A bacterial net was observed growing in the e-waste surface, absorbing mostly iron and solubilizing 17 g/l of copper.

GRAPHICAL ABSTRACT



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ABSTRACT

The bacteria isolated from *Hymeniacidon heliophila* sponge cells showed bioleaching activity. The most active strain, Hyhel-1, identified as *Bacillus* sp., was selected for bioleaching tests under two different temperatures, 30 °C and 40 °C, showing rod-shaped cells and filamentous growth, respectively. At 30 °C, the bacteria secreted substances which linked to the leached copper, and at 40 °C metallic nanoparticles were produced inside the cells. In addition, infrared analysis detected COOH groups and linear peptides in the tested bacteria at both temperatures. The Hyhel-1 strain in presence of electronic waste (e-waste) induced the formation of crust, which could be observed due to bacteria growing on the e-waste fragment. SEM-EDS measurements showed that the bacterial net surface was composed mostly of iron (16.1% w/w), while a higher concentration of copper was observed in the supernatant (1.7% w/w) and in the precipitated (49.8% w/w). The substances linked to copper in the supernatant were sequenced

* Corresponding author at: LSCP, Chemical Engineering Department, University of São Paulo (USP), Av Prof Lineu Prestes 580, Block 21, CEP 05508 910 São Paulo—SP, Brazil.
E-mail address: isoquir@gmail.com (E.E. Rozas).

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by MALDI-TOF-ms/ms and identified as macrocyclic surfactin-like peptides, similar to the basic sequence of Iturin, a lipopeptide from *Bacillus subtilis*. Finally, the results showed that Hyhel-1 is a bioleaching bacteria and cooper nanoparticles producer and that this bacteria could be used as a copper recovery tool from electronic waste.

1. Introduction

Technological progress stimulates the use of electronic devices. At the same time, the lifespan of these equipments has been decreasing rapidly. This situation has generated a large volume of e-waste, leading to a critical problem of disposal [1]. The natural leaching of hazardous substances present in e-waste may be responsible of the contamination of groundwater by heavy metals and its incineration, on the other hand, produces toxic gases [2]. The traditional method of metal recovery from e-waste by "smelting" is a process which produces dioxin and furan from plastic components. An alternative route is hydrometallurgy, which uses solvents for solubilization of materials that constitute electronic equipment [3]. Chemolithotrophic bacteria such as *Acidithiobacillus ferrooxidans*, frequently used to primary ores bioleaching, may be an alternative process to extract the major metals component, such as copper, nickel and other elements present in e-waste [4].

Copper bioleaching of primary ores is carried out at low pH (1–2), producing an accelerated mineral corrosion by the bacteria and causing the metal cations to remain in solution [5]. The most studied bioleaching microorganism is *A. ferrooxidans*, however, other genera such as *Acidianus, Metallosphaera, Sulfobacillus, Sulfolobus, Leptospirillum, Thiobacillus* and *Ferroplasma* are usually present in the commercial bioleaching processes [6]. In contrast, few records related to bioleaching in alkaline or neutral pH have been published. One example is the dissolution of polymetallic nonferrous material treated with a cell-free medium from a *Bacillus* culture [7]. Specifically, publications about bioleaching of copper of e-waste in alkaline media were not found in the literature survey.

Regarding the metabolic mechanism, bioleaching bacteria generate energy by an indirect electron transfer using redox-active compounds released by bacteria, without physical contact between bacterial cell membrane and mineral surface [9,10]. *Acinetobacter calcoaceticus*, for instance, uses a self-excreted redox compound similar to pyrroloquinoline quinine to generate energy [11]. These extracellular substances are metabolic products, such as glycoproteins, polysaccharides, proteins, cellulose, lipids and glycolipids, that offer cation binding sites, thus providing protection against heavy metal [12,13]. Another way to neutralize heavy metal toxicity on the cells is through nanoparticle formation, a mechanism that reduces the ionic force of the metals, enclosing metallic nanoparticles in vesicles that can later be released [14]. This transition between metal ions and non-toxic forms has been suggested as a mechanism to survive in contaminated environments [15].

Biological processes leading to accumulation of metals and/or nanoparticle formation have also been observed in sponges (phylum Porifera). Laboratory experiments showed that metal concentration outside the sponge cells were lower than the internal one, indicating that *Halichondria panicea* accumulated copper, zinc or cadmium when exposed to these metals [16]. Tissues of *Suberites domuncula*, for example, showed cadmium values 24 times higher than the concentration observed in samples of seawater [17]. Meanwhile, whole extracts of *Acanthella elongata* exposes to $AuCl_3$ and $AgNO_3$ solutions produced nanoparticles with a coat of amine [18,19]. Similarly, chitin isolated from *Aplysina aerophoba* promoted the formation of Fe_2O_3 nanocrystals [20]. However, the biological mechanism of the mineralization or nanoparticle formation in sponge until now was not reported.

Sponges are well known to be hosts for a large community of microorganisms, such as bacteria and fungi, and some of these microbes are probably host-specific, representing up to 50% of the sponge tissue volume [21]. Copper-exposed *Rhopaloeides odorabile* showed a significant reduction in bacteria diversity, mostly remaining the *Pseudomonas* genus, known to be resistant to high copper concentrations [22–24]. Thus, it is possible that the accumulation of metals and nanoparticle formation by sponge tissues are a result of bacteria-sponge association [25,26]. In view of this possibility, this study aimed to test the ability of bacterial strains isolated from sponge cells to interact with copper present in e-waste.

2. Material and methods

2.1. Sponges

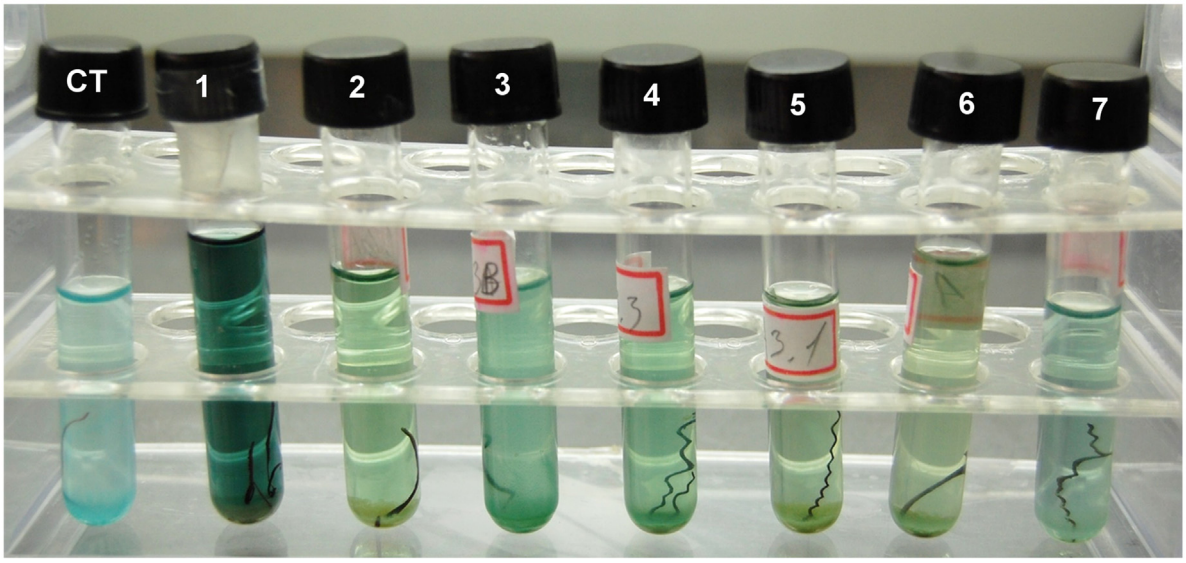
Samples of the marine sponge *Hymeniacidon heliophila* (Halichondrida: Demospongiae) were collected at the São Vicente region (23°58′60″ N, 46°22′0″ E), in southeastern Brazil. The specimens were placed in plastic bags and transported in cooled boxes (18 °C) to the laboratory, where they were maintained in a marine aquarium. Taxonomic identification was carried out by the authors using standard methods.

2.2. Sponge cells

The sponges were dissociated in a calcium and magnesium-free artificial seawater with EDTA (CMFSW + E: 460 mM NaCl, 7 mM Na_2SO_4, 10 mM KCl, 10 mM HEPES, 2.5 mM EDTA, pH 8.2) according to Custódio et al. [27]. Tissue pieces of approximately 2 mm wide on each face were maintained in CMFSW + E for 30 min in a low speed rotatory shaker [28]. Then, the supernatant was discarded and the remaining pieces were shaken for 45 min in fresh CMFSW + E. The supernatant was filtered through a 40 μm nylon mesh, centrifuged at 250*g* for 10 min and re-suspended in sterile natural seawater supplemented with kanamycin 100 mg l^{-1}, gentamicin 50 mg l^{-1}, tetracycline 10 mg l^{-1} and phenol red (16 mg l^{-1}). The medium was changed daily for one week until the cells formed aggregates.

2.3. Isolation of bacteria from the sponge cells

The aggregates were distributed in 24-well plates, with 2 ml of copper solution in each well. As controls, four wells received only the solution, without sponge cells. The copper solution was prepared dissolving $CuSO_4 \times 5H_2O$ in sterile seawater and used in concentrations of 0.1, 0.4, 0.6, 0.8 and 1 mg ml^{-1}. After incubation in an orbital shaker at 30 °C for 24 h, the aggregates were centrifuged at 10,000*g* for 3 min, the supernatant was discarded and the sponge cells lysed in a 0.1% Triton X-100 solution. Samples of the lysate (0.5 ml) were diluted serially in 4.5 ml of sterile seawater and 0.1 ml of each dilution was disseminated in Brain Heart agar 2% NaCl (BHI 2% NaCl). The colonies were then isolated and incubated in Brain Heart infusion 2% NaCl for one week and disseminated in new agar plates to verify the purity of each strain. Finally, the bacteria copper



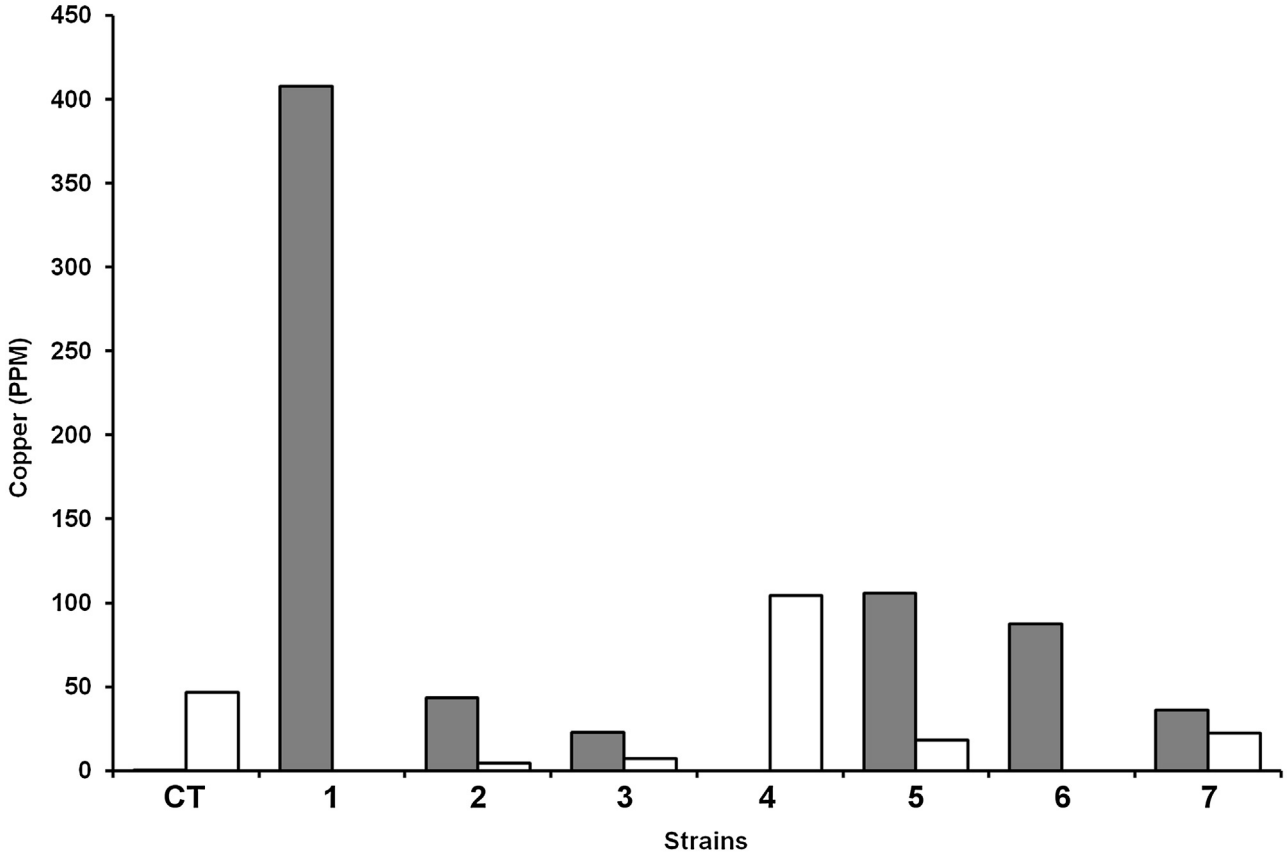


Fig. 1. Bacteria isolated from the sponge *H. heliophila*. A) Seven strains of bacteria obtained from sponge cells after the initial incubation with CuSO4 and cultivated in presence of copper wires. B) Copper concentration in solution (gray bars) and in the precipitated (white bars) of each culture. The precipitated detected in the controls have abiotic origins.

tolerance was tested incubating each isolated strain in two different concentrations of $CuSO_4$ (1 and 10 mg/ml) in seawater.

2.4. Bioleaching activity

One milliliter (5 mg dry weight) of each strain isolated from the sponge cells was added to 4 ml of seawater supplemented with 1% dextrose (SWD) together with a copper wire (CuW, 200 ± 2 mg), sterilized in an open flame, and incubated for 15 days at 30 °C without shaking. The control was prepared adding a piece of CuW to 5 ml of SWD without bacteria, maintaining the same culture conditions. At the end, the culture confluence was measured by optical density at 600 nm in a spectrophotometer. Samples of the bacteria and supernatants were stored at −20 °C until analysis.

The copper released in the media was measured by absorption atomic spectroscopy (AAS) AA-700 (Shimadzu). The strain that

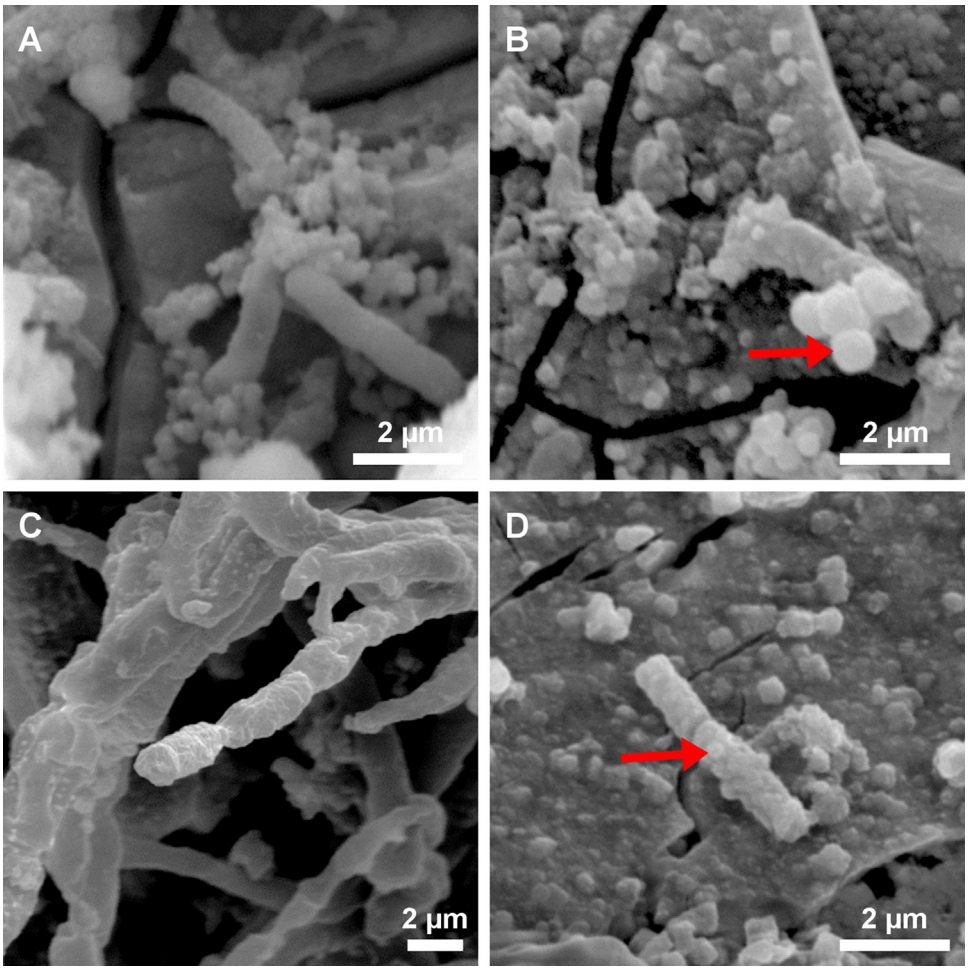


Fig. 2. Scanning electron microscopy of Hyhel-1 incubated in SWD medium in presence of cooper wire in two temperatures, showing the different life forms. A and B) As free rod-shaped cells at 30 °C. C and D) As filamentous cells at 40 °C. Arrows indicating vesicles secreted by bacterium.

showed the higher activity was then selected for further characterization and cultivated in BHI 2% NaCl to provide the initial inocula. Five milliliters of the culture (25 ± 0.1 mg dry weight) were incubated together with 504.4 ± 5.0 mg of CuW in 300 ml of SWD at 30 or 40 °C for 15 days without shaking. Controls for both temperatures were prepared incubating CuW in SWD without bacteria. Previously to the AAS analysis, the supernatant was centrifuged at 10,000*g*, filtered through a 0.22 μm membrane, digested three times with 1 ml of fuming nitric acid and diluted in 5 ml of nitric acid 5%. Samples of the bacteria and the copper wire fragments were fixed in glutaraldehyde 2.5% to be analysed through electronic microscopy. The bacterial cultures were also dried in speedyvac and analyzed in an infrared spectrometer (ATR systems) IR Prestige21 (Shimadzu).

2.5. Bacteria identification

Bacteria identification by mass spectrometry was performed in a MALDI-TOF-MS Ultraflextreme (BIOTYPER, Brueker Daltonics) using α-Cyano-4-hydroxycinnamic acid as matrix. Bacteria samples were resuspended in ethanol 75%, pelleted at 16,200 g for 2 min and then the liquid was removed. Next, the pellet was resuspended in 50 μl of formic acid 70%, added with 50 μl of acetonitrile 1% TFA and sonicated for 5 min. The extract was centrifuged at 16,500*g* for 3 min and 0.8 μl of the supernatant was spoted in MALDI-TOF target and dried. Accordingly, 0.8 μl of saturated matrix solution was added to each spot. The obtained were searched in the database of the manufacturer. For the bacteria identification by DNA sequence analysis, the 16S region was extracted from an assembly of the isolated strain (GenBank accession number: KU942606). Alignments to 16S sequences of *Bacillus* sp. that were the best hits after Blast analysis [29], (GenBank *Bacillus methylotrophicus* strain YJ1114: CP011347.1, *Bacillus* sp. SDLI1: CP013950.1, *Bacillus* sp. Pc3: CP010406.1, *Bacillus subtilis* strain ATCC 19217: CP009749.1, *Bacillus amyloliquefaciens* subsp. *plantarum* SQR9: CP006890.1, *Bacillus amyloliquefaciens* strain MBE1283: CP013727.1, *Bacillus polyfermenticus* strain GR010 16S ribosomal RNA gene: DQ659145.1, *Bacillus atrophaeus* strain NRS 1221A: CP010778.1, *Bacillus subtilis* strain Bs916: CP009611.1, *Bacillus subtilis* subsp. *subtilis* str. 168: CP010052.1, *Lactobacillus acidophilus* 30SC: NC_015214.1, *Haloanaerobacter chitinovorans*: U32596.1, *Heliobacterium sulfidophilum* strain BR4: NR_025090.1, *Thermodesulfobium narugense* DSM 14796: NC_015499.1,) was carried out by CLUSTAL using distance matrix and Jalview V2 for alignment editing and visualization [30,31].

2.6. Electron microscopy

Scanning electron microscopy (SEM) analysis of the copper wires and controls was conducted with an accelerating voltage of 15 KV in samples previously gold coated. Transmission electron microscopy (TEM) analysis was carried out in bacteria collected from remaining parts after centrifugation at 10,000*g* of the cultures supernatant with and without the copper wire. The samples were

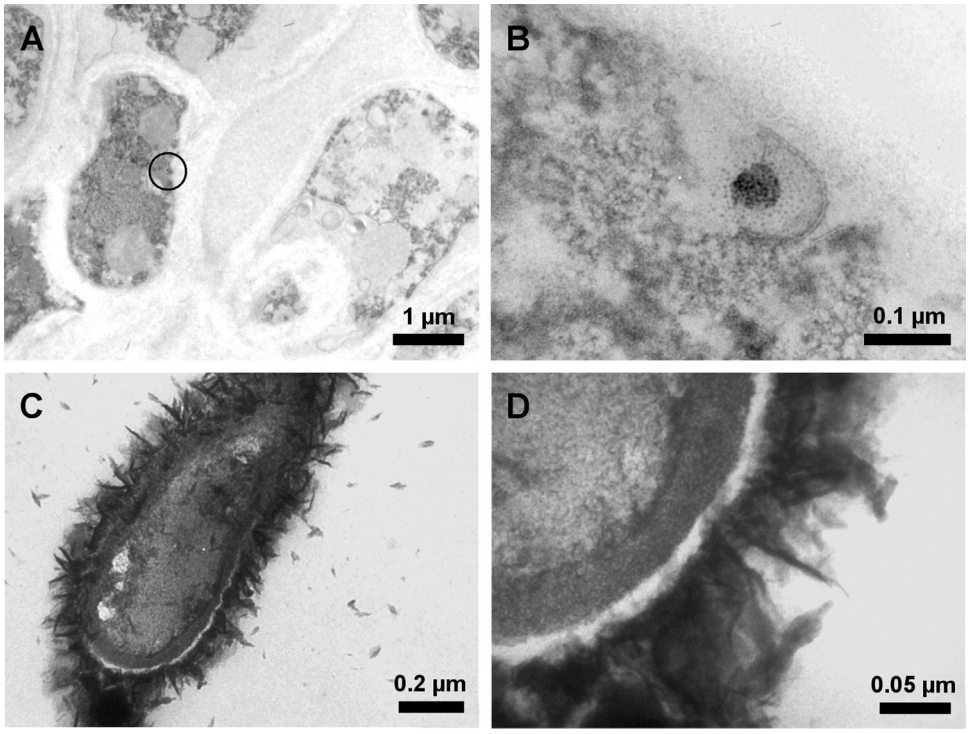


Fig. 3. Transmission electron microscopy of Hyhel-1 incubated in SWD medium in presence of copper wire in two temperatures. A) The strain at 40 °C producing nanoparticles (circle) inside in vesicles. B) Detail of the vesicle enclosed in the circle in A. C) Bacteria exposed to copper wire at 30 °C showed an increased presence of substances covering the cells and released to medium. D) Detail of the cell wall in C.

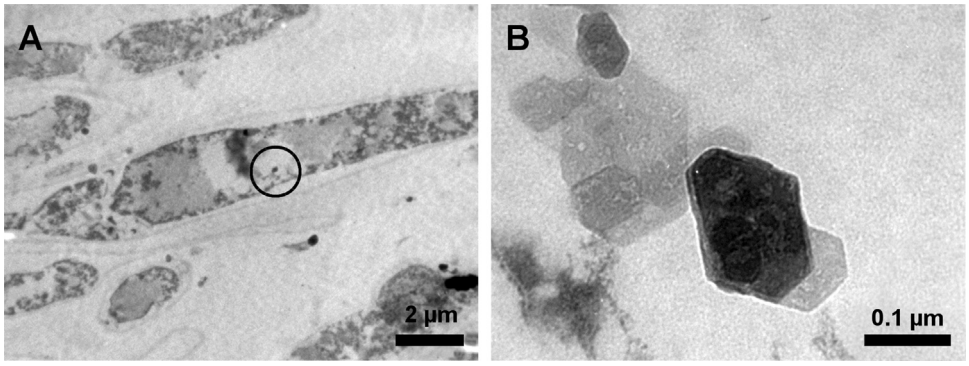


Fig. 4. Intracellular microcrystals formation by Hyhel-1 exposed to copper wire at 40 °C in SWD medium. A) Whole cell image with the microcrystals (circle) formed by nanoparticles. B) Detail of the circle in A.

washed three times in PBS buffer (pH 7.0) and embedded in resin (Embed-812, EMS). Ultrathin sections (50–70 nm) were stained with uranyl acetate and lead citrate for 30 and 5 min, respectively, and then examined in an electron microscope (EM900, Zeiss).

Analysis of the nanoparticles by energy-dispersive X-ray spectroscopy (EDX) was prepared by extracting in formic acid the bacteria incubated at 40 °C in presence of CuW. The copper can be recovered from inside the cells by extracting the nanoparticles using formic acid. When the sample was put in acid, the cells were lyzed and the nanoparticles were exposed. The nanoparticles were then dried under vacuum, rehydrated in Milli-Q water and centrifuged at 10,000*g* at 4 °C for 20 min. The supernatant was collected and centrifuged again under the same conditions. The supernatant containing Cu nanoparticles was dropped onto carbon coated grids and left standing for 24 h in the vacuum chamber at 60 °C prior before the TEM analysis.

2.7. Bioleaching of e-waste

Grounded e-waste obtained from printer motherboards (PMB) was provided by the Recycling, Waste Treatment and Extractive Metallurgy Laboratory at the University of São Paulo (LAREX-USP). First, PMB components such as the capacitor, resistors, and sockets were removed. Second, the PMB was cut off by a 9 mm grid knives mill and crushed by a 2 mm grid hammer mill. The samples were then leached by aqua regia and followed by ICP analysis. The ICP results showed the PMB's elemental composition (% w/w) to be: Cu (32.5), Al (3.2), Fe (1.42), Sn (0.96) Ca (1.13), Zn (0.64) Ni (0.34), Ba (0.27), Sb (0.01), Ag (0.31), Na (0.05), K (0.03), Au (0.004) and 2.57% of other elements.

Pure cultures of the selected strain were used as inoculum and all tests were performed in 250 ml flasks using BHI 2% NaCl diluted 50% in Milli-Q water. To obtain metal-adapted cultures, 190 ml of the mentioned above media was prepared in 300 ml Erlenmeyer flasks containing 5 g of e-waste and sterilized by autoclaving for

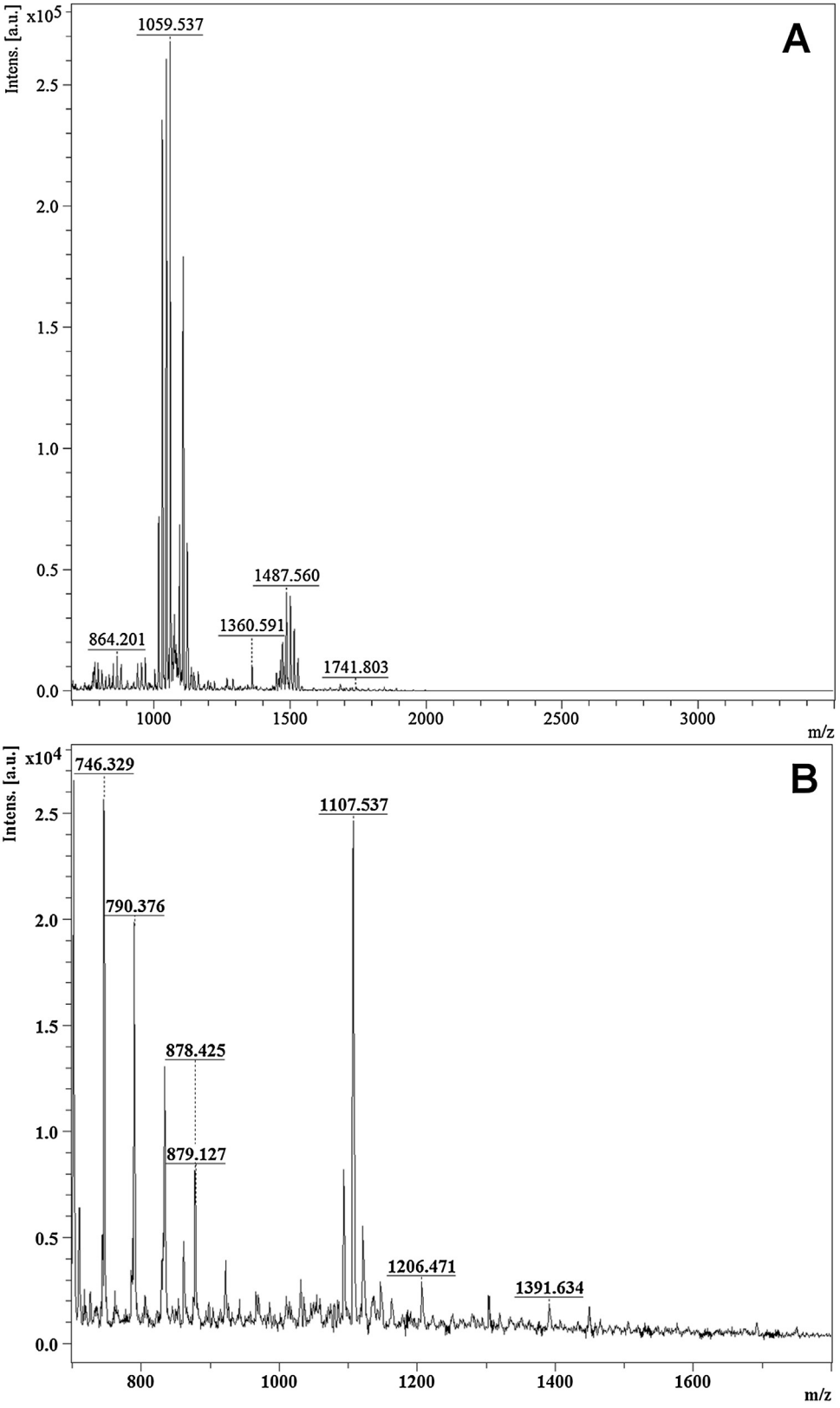


Fig. 5. MALDI-TOF-MS analysis of SepPak C18 ethanolic fraction from supernatant of cultures of Hyhel-1 incubated with e-waste. A) Control. B) Bioleaching.

30 min. The flasks were inoculated with 10 ml (500 mg dry weight) of the selected strain and incubated at 30 °C in an orbital shaker for 3 days. After incubation, the inocula (10 ml) were transferred to new flasks containing 190 ml of medium and 5 g of previously autoclaved e-waste. Controls were prepared in flasks containing 200 ml of medium and 5 g of e-waste without bacteria. All flasks were incubated at 30 °C in a shaker at 200 RPM during 45 days. Fragments of e-waste incubated for 7 and 45 days were observed in SEM to ver-

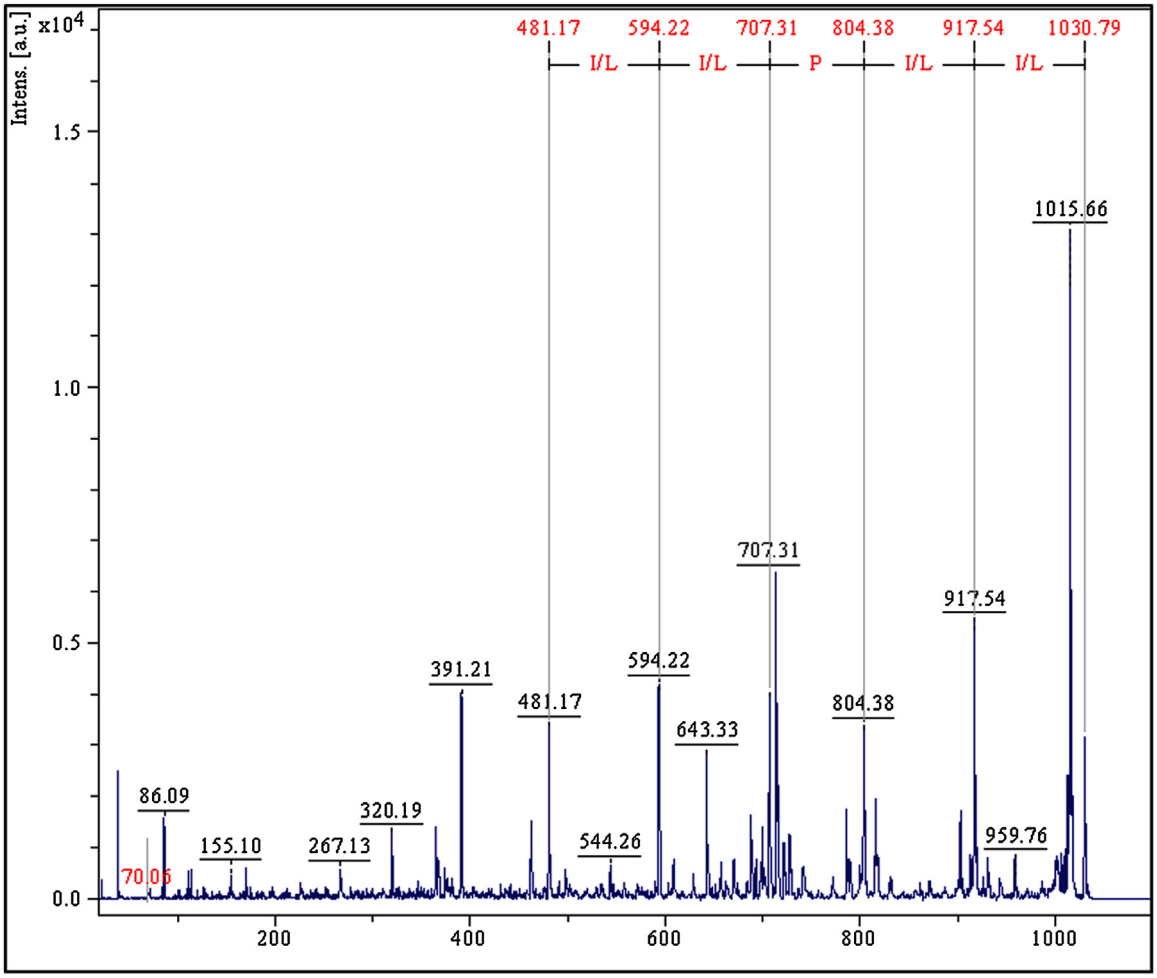


Fig. 6. Mass spectrometry sequencing of peptide 1044 *m*/*z* from supernatant of control cultures of Hyhel-1.

ify the bacterial growth in the surface. At the end, the supernatants were separated from the precipitates by sedimentation. The supernatants were analyzed directly by EDX Epsilon3-XL (PANanalytical) and the precipitates were dried under reduced pressure and manually grounded to a fine powder using a mortar with pestle before analysis.

2.8. Peptide extraction and sequencing

Peptide sequencing was performed using MALDI-MS/MS analysis. Supernatant samples from the test and control cultures were loaded in Sep-Pak C18 columns and washed with Milli-Q water. The columns were then eluted with 5 ml of ethanol 50% and the retained substances recovered with 5 ml of ethanol (HPLC grade). Aliquots (0.6 μl) of the ethanol fractions were placed in a MALDI-TOF target, air dried and covered with 0.6 μl of α-Cyano-4-hydroxycinnamic acid matrix. The spectra were recorded using a LIFT mode in Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics) by selecting appropriate precursor ions within a window range of ±3 Da. The Flexanalysis software (Bruker Daltonics, ver. 3.4) was used for the MALDI-MS data analysis.

3. Results and discussion

Seven bacterial strains, named Hyhel-1 to Hyhel-7, were isolated from sponge cells, which were incubated with 1 mg/ml $CuSO_4 \times 5H_2O$. Hyhel-1 strain showed the highest bioleaching activity of the seven bacteria incubated with copper wires (CuW), achieving the concentration of 407.6 mg/l of copper in the liquid phase after seven days of incubation (Fig. 1A, B). In this experiment, Hyhel-1culture reached confluence in 24 h (OD_{600} = 2.4), while the Hyhel-4 and Hyhel-6 reached the same stage in 48 h and the other strains after 96 h. Bioleaching activity studied in this work was development in seawater and in BHI 2% NaCl, with initial pH of 8.0 and 7.4, respectively, reaching in the end of the process pH of 5.3 in both cases. This pH data differs from strong acidic environment (pH 1–2) used in traditional bioleaching methods, as described to copper extraction from circuit boards using *Acidithiobacillus ferrooxidans* [2,32], or thermophilic bacteria [33].

Thus, considering its highest leaching activity and fast growth, the Hyhel-1 strain was selected as a model to study the bioleaching process in this work. Taxonomic identification of Hyhel-1 was conducted by MALDI-TOF mass spectrometry and through the 16S DNA sequencing of a fragment obtained from a partial genome assembly. The sequence was deposited in Gen Bank (accession number: KU942606). Both MALDI-TOF and 16S DNA showed a high level of identity to *Bacillus* sp and the closest species were *Bacillus methylotropicus* (CP011347.1) and *Bacillus subtilis* (CP009749.1) (Supplementary Fig. 1).

Hyhel-1 bioleaching experiments were conducted in presence of copper wire at 30 °C and 40 °C, showing differences in the supernatant color, as well as, in the quantity of precipitated material. The strain incubated at 30 °C showed a green precipitate and a blue supernatant, while the same strain incubated at 40 °C showed

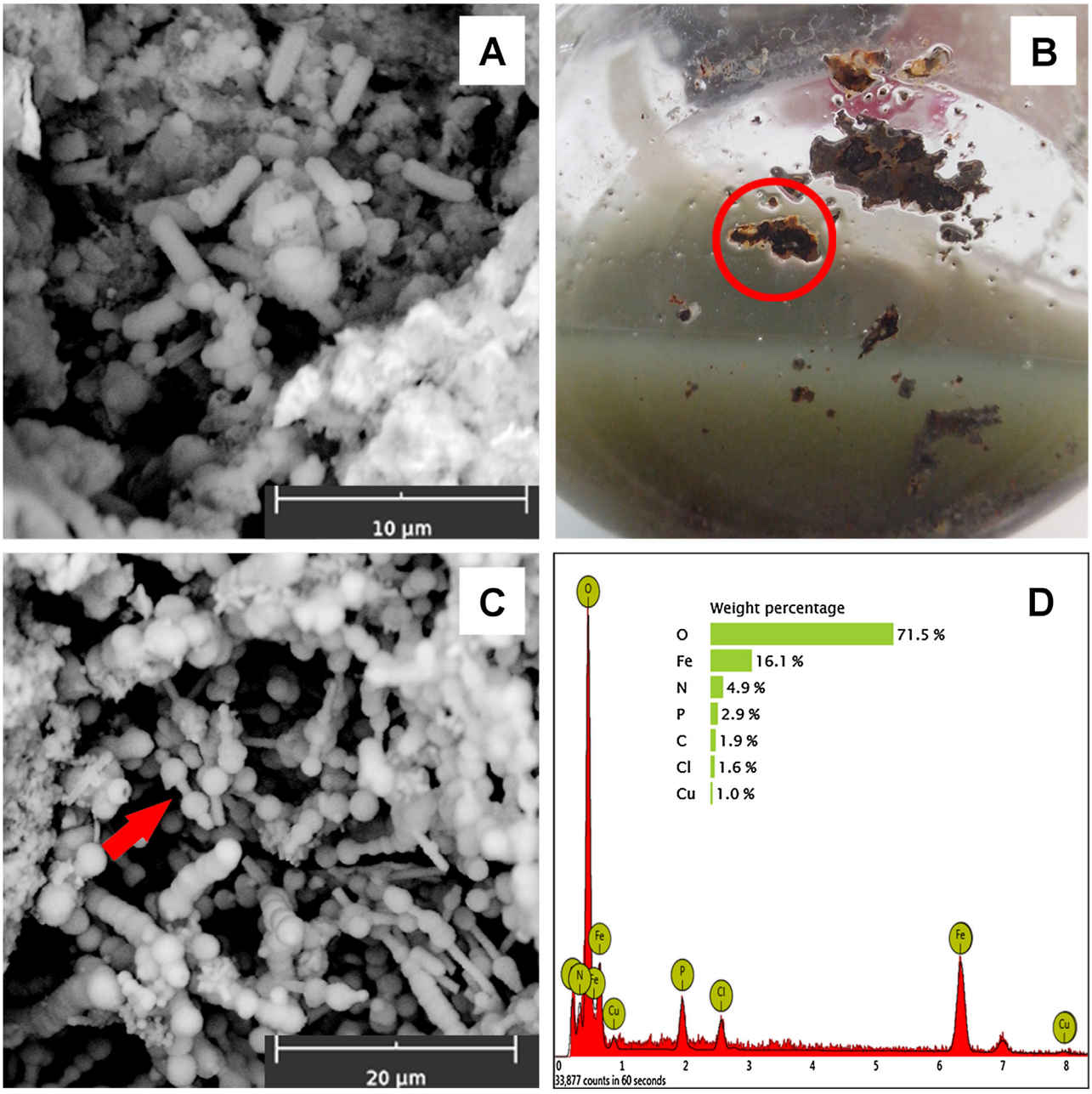


Fig. 7. Bioleaching of e-waste by Hyhel-1. A) Bacteria growing in the surface of e-waste particles after seven days of culture. B) Bioleaching culture after 45 days. The circle encloses one metallic crust attached to the bottom of the flask. C) Electron microscopy of the metallic crust showing the interconnected cells. The arrow indicates the position analyzed by EDX. D) Elementary composition obtained by EDX of the point in the bacterial net in the metallic crust.

a brown precipitate and clear brown supernatant. The amount of copper released in the medium was slightly lower at 30 °C cultures (680 ± 40 mg/l) in comparison to cultures at 40 °C (782 ± 65 mg/l). The precipitate (≅227 and 293 mg respectively) showed 50.1% (501 g/kg) of copper at 30 °C and 43.3% (433 g/kg) in the 40 °C cultures. These results showed that leached copper was bounded by Hyhel.1 cells and most of the copper wire was released into the medium as ion in solution.

The SEM analysis of Hyhel-1 at 30 °C showed vesicles being released from the cells (Fig. 2A, B), while at 40 °C only a few vesicles over the metal surface were observed (Fig. 2C, D). Furthermore, it could be noticed that Hyhel-1 displays different morphology at each temperature: rod-shaped free cells at 30 °C and filamentous organisms at 40 °C. These morphologies were previously reported for *Sphaerotilus natans*, changing from filamentous sheaths to rod-shaped single cells under anoxic conditions with Fe(II) and nitrite addition, seen as "ball-shaped" forms on the cell surfaces [34]. Hyhel-1 showed similar structures on the cells surface, although the cultures were realized under aerobic conditions in presence of CuW without Fe(II) or nitrate addition. These facts suggest that these structures could be an adaptation of Hyhel-1 to release metals absorbed by the cells in order to neutralize their toxicity, and not a reaction to a specific compound [35]. However, Hyhel-1 released vesicles produced by the cells and leached the CuW, disagreeing with the vesicles formation as neutralizing mechanism, since the Hyhel-1 survived in a high concentration solution of copper.

The TEM analysis of Hyhel-1 in presence of CuW at 30 °C and 40 °C showed morphological differences in the bacterial cells and their behavior in respect to the copper allocation. Bacterium incubated in presence of CuW at 30 °C showed an electro-dense

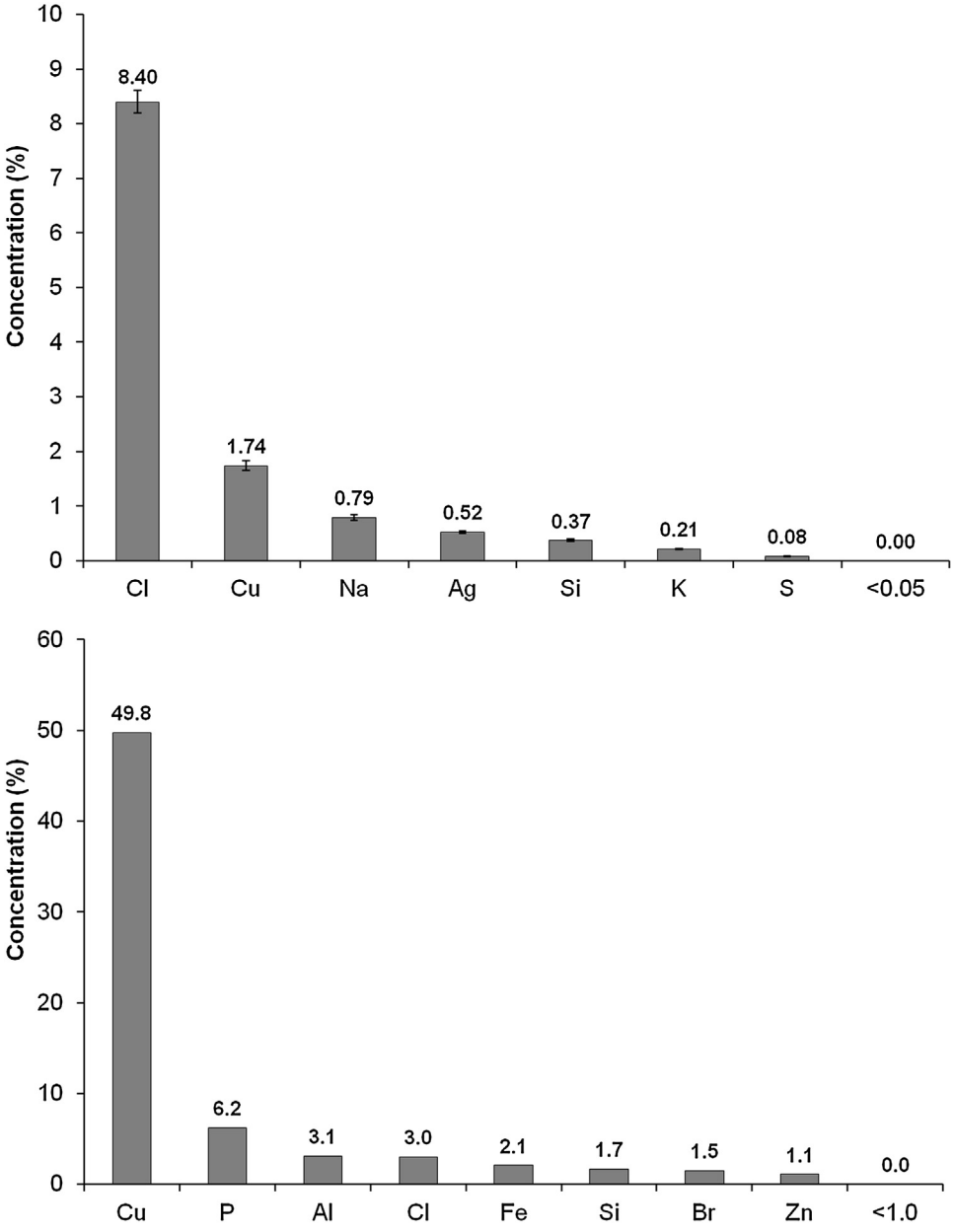


Fig. 8. Relative concentration of compounds measured by energy-dispersive spectroscopy in the e-waste bioleached by Hyhel-1 cultures. A) Supernatant. B) Precipitated.

peptidoglycan layer and an external "*zona pellucida*"-like membrane, covered by substances that absorbed the copper released to the medium. Differently, Hyhel-1 incubated at 40 °C showed clusters of nanoparticles inside the cells and in vesicles, presenting an external membrane without ornaments (Fig. 3). A similar accumulation of nanoparticles was observed in plancktonic cells of *Geobacter* sp., which showed aggregated ferric oxyhydroxide nanoparticles attached to the external membrane [36]. Hyhel-1 produced in turn the aggregated inside the cells, enclosing the nanoparticles with a membrane. After the extraction of nanoparticles using formic acid they were analyzed by TEM-EDS. The TEM-EDS analysis of these nanoparticles showed that they are composed of copper and reach dimensions smaller than 1 nm (Supplementary material 2). In this condition, it could also be observed that the extracellular material binding to copper was internalized and grouped inside the cells, producing a nucleus that seems to be the starting point to nanoparticles formation (Supplementary material 3). These nuclei assembling microcrystals suggest that copper nanoparticles inside the cells help the formation of microcrystals (Fig. 4), that could be grown by nanoparticles addition. This type of crystal formation was previously reported to chemical conversion of Cu_2O nanoparticles into Cu crystals, using polyacrylamide as surfactant to extract the Cu^{+2} from Cu_2O and ascorbic acid as reducing and nucleating agent [37]. Surfactant and reducing agents are indeed present in *Bacillus* [38,39], suggesting that Hyhel-1 can bind the metal with extracellular substances and use the ascorbic acid present inside the cells to form nanoparticles, later grouped into crystals. The FTIR analysis of Hyhel-1 exposed to CuW (Supplementary material 4) showed absorption peaks at around 1036–1076 cm^{-1} in leaching cultures, indicating an involvement of COOH groups in the linked metal. Similar observations demonstrated that carboxyl group of extracellular polymeric substances of *Acinetobacter junii* was the binding site for divalent metal ions [40]. Singularly, Hyhel-1 cultures incubated with CuW at 30 °C showed a higher absorption peak in 3333 cm^{-1}, indicating the involvement of simple peptides in the leaching process. These data suggest that

the products observed on the surface and released by the cell in response to CuW at 30 °C were also peptides. For this reason, it was suggested that the nanoparticles formation and the copper bioleaching in the supernatant are associated to peptides that after absorbing the copper cations were then internalized and concentrated to form the metallic nucleus.

The MALDI-TOF mass spectrometry analysis showed that peptides of *m*/*z* 1017.5; 1031.4; 1045.5; 1,053.4;1059.5 and 1067.4 present in the control supernatant were not observed in the cultures with CuW (Fig. 5). This suggests that the metal was absorbed by these peptides released by Hyhel-1, producing a green precipitate. This was corroborated by FTIR analysis of CuW cultures incubated at 30 °C, confirming that the peptides produced by Hyhel-1 absorb copper. *De novo* sequencing of parent ion of *m*/*z* 1,017.5; 1,045.5; 1,059.5 e 1,074.5 showed that these peptides shared the same basic sequence I/L-I/L-P-I/L-I/L (Fig. 6). The sequencing data suggest that the peptides that precipitated by linking to metal were macrocyclic surfactin-like peptides, a characteristic lipopeptide present in *B. subtilis* [41]. These substances, described as biosurfactants have been previously used to remove heavy metals from contaminated soil [42]. Moreover, it is possible that bacterial extracellular substances bound to copper could be a lipopeptide structurally similar to Iturin. The molecules of *m*/*z* 1,031.4; 1,045.5 and 1,059.5 differed in their masses by 14 Da, while 1,053.4 and 1,067.4 only by 2 Da from Iturins, suggesting them to be members of the same family [43].

In this study, Hyhel-1 was also exposed to e-waste to evaluate its behavior in contact to this material. The bioleaching tests showed that after seven days Hyhel-1 colonized the surface of the e-waste fragments and after 45 days constructed a crust linking the fragments with a bacterial net build by connections between cells (Fig. 7). Similar crusting behavior was observed in the marine sulfate-reducing bacteria *Desulfopila corrodens*, isolated from a biogenic mineral crust formed during iron corrosion [44]. The bacterial net formed by Hyhel-1 was similar to the one observed in gold biomineralization by *Cupruavidus metallodurans*, which showed extracellular projection formed by exopolymers or nanowires with nanoparticles deposition in the surface [45]. Nevertheless, the Hyhel-1 bacterial projections seem to be formed by successive cell divisions without separation, differently from the nanowires in *C. metallodurans*.

SEM-EDS measurements showed that the bacterial net surface was composed mostly of iron (16.1% w/w) (Fig. 7D), while EDX analysis of the supernatant indicated that chloride was the most abundant element (8.40%), followed by copper (1.74%). In contrast, the precipitate showed a high copper concentration (48.9%) and low chloride concentration (3.0%) (Fig. 8). Considering that copper is the main metal component of the printer motherboard, traditional bioleaching process used in copper mine ores has been previously applied to recover metal from e-waste [46]. However, in the spite of new approaches such as using a mixed culture of biosurfactant-producing bacteria and *Acidithiobacillus* sp., the results showed an increase of copper removal rate has only in acidic medium [47]. Even so, Hyhel-1 showed to be able to remove 4.86% of the copper initially present in the printer motherboard while pH was only reduced to 5.3. Another highlight was the surfactant-like produced by Hyhel-1. Karwowska et al. [47] showed that the surfactant released by *Bacillus subtilis* co-cultured with *Acidithiobacillus* sp. increases copper rate removal, which suggests that Hyhel-1 is a leaching bacteria capable of enhancing its bioleaching activity by producing surfactant that binds the copper in solution.

Furthermore, producing copper nanoparticles directly from e-waste as performed by Hyhel-1 is a method still not described in literature. Usually, nanoparticles made from e-waste are produced by chemical methods such as reduction, thermal decomposition or electrochemical synthesis [48]. Synthesis of nanoparticles using green methods had already been performed by using cells or extract of plant, bacteria, fungi or algae to reduce and nucleate ionic metal solutions, but not directly from e-waste [49–51]. Considering these results, it is possible to suggest that the lipopeptides produced by Hyhel-1 were responsible for releasing the copper from the e-waste and bringing it into the bacteria cells in order to form nanoparticles.

4. Conclusion

The experiments carried out in this study revealed that, Hyhel-1, a *Bacillus* isolated from the marine sponge *H. heliophila*, was able to leach electronic waste and to produce copper nanoparticles. These leaching process was started in neutral pH environment (8.0; 7.3), reaching in the end of the process pH of 5.3 in both cases, indicating that Hyhel-1 used a different mechanism than acidophilic bacteria. Peptide released by bacteria (1,017.5; 1,031.4; 1,045.5; 1,053.4;1,059.5 and 1,067.4 *m*/*z*) were involved in the copper metal leaching, absorbing ion copper and being incorporated into the cells for nanoparticle production. The amount of these peptides was increased in the presence of copper wire suggesting protection activity. Thus, the Hihel-1 strain offers a new alternative to recover metals from e-waste without using solvents or acidification, which increase costs.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2017.01.037.

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