Biomining of Lunar regolith simulant EAC-1A with the fungus Penicillium simplicissimum

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Article

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Abstract

On a future lunar habitat, acquiring needed resources in situ will inevitably come from the Lunar regolith. Biomining-the use of microorganisms to extract metals from the regolith-is sustainable and energy-efficient, making it highly promising for space exploration applications. Given the extensive use of filamentous fungi in industrial biotechnology, we investigated the ability of the fungus Penicillium simplicissimum to extract metals from the European Astronaut Centre lunar regolith simulant 1 (EAC-1A), which will be used as the analogue soil at the European Lunar Exploration Laboratory (LUNA) facility at the European Space Agency (ESA) and German Aerospace Centre (DLR) site. Biocompatibility tests demonstrated P. simplicissimum tolerance to high concentrations of EAC-1A regolith (up to 60 %), both on Earth gravity and Lunar simulated gravity. A fungal bioleaching setup was developed using a low nutrient medium, that allowed the fungus to extract metals from EAC-1A over the course of 2 weeks, including aluminium, iron, magnesium and calcium, among others. Metal recovery from the leachate achieved a promising average of 10 ± 3 g/L of metal powder. Our study demonstrates fungal biomining as a promising in situ resource utilization (ISRU) approach to be used in future missions to the Moon.

Introduction

In future lunar exploration missions, transporting resources from Earth will be one of the main challenges[1]. To reduce the logistical challenge of resupply using Earth's resources, In situ Resource Utilization (ISRU) approaches will play a critical role in obtaining essential supplies in a cost-effective and sustainable manner[2,3]. In a Lunar exploration scenario, raw materials such as the soil, are widely available and can be explored for use in various areas, from life support systems (water-H_2O) to fuels and propellants (hydrogen-H_2)[4]. In particular, the Lunar regolith - a superficial layer of unconsolidated materials – is expected to be a primary source of *in situ* resources, mainly through mining and extraction technologies[5]. Samples recovered from Apollo's missions reveal that Lunar regolith comprises a vast variety of metals (Table 1), for example, iron, aluminium, or titanium[6]. These metals can be mined *in situ* and be used for the fabrication of equipment, construction materials, as well as to integrate devices for power generation[7]. Among the various power generation technologies available, solar cells stand out as a promising option for lunar exploration, as they can be produced entirely *in situ* using highly abundant materials such as silicon, aluminium, and other metals found in lunar regolith[6,8].

Table 1
Abundance (wt. %) of chemical compounds with metallic elements from the sample 70017 recovered from the Moon during the mission Apollo 17, and EAC-1A, adapted from Duncan et al., 1974, and Engelschiøn *et al.*, 2020.

COMPOUND	ABUNDANCE IN APOLLO 17	ABUNDANCE IN EAC-1A
SiO_2	38.37	44.35
FeO	18.71	11.35
TiO_2	12.83	2.4
CaO	10.43	10.8
MgO	9.41	11.9
Al_2O_3	8.78	12.6
Na_2O	0.43	2.9
MnO	0.28	11.9

To prepare astronauts for human exploration of the Moon, the European Space Agency (ESA) and German Aerospace Center (DLR) are currently constructing the European Lunar Exploration Laboratory (LUNA), in Cologne, Germany. The LUNA facility will work as a testbed to explore the technologies that might ease human settlements beyond Earth. To fill LUNA's 660 m^2 area with lunar regolith simulant material, the European Astronaut Centre lunar regolith simulant 1 (EAC-1A) - a basaltic sandy silt from a quarry located in the Siebengebirge volcanic field - is considered as a large-volume source of material[9]. EAC-1A is comparable with widely used lunar regolith simulants, namely JSC-1A, JSC-2A, NU-LHT-3M, DNA and FJS-1, each with slightly varying properties such as the grain size or composition[9–11].

While mining is fundamental for our civilization, traditional methods are undesirable for sustainable space exploration, due to high energetic costs and environmental damages[12]. Alternative mining approaches such as molten salt electrolysis or the FFC process, are currently being studied to liberate metals from the lunar regolith, however these processes are still energetically intensive[13]. In contrast, the use of microorganisms to extract desirable metals, i.e. biomining, is a sustainable and energy-efficient biotechnological alternative to obtain metals for human space exploration[14]. On Earth, biomining is responsible for up to 25% of copper and 5% of the total gold mining[15], and can be used in the recovery of metallic elements from electronic waste[16].

Filamentous fungi are notably known for their role in bioleaching: a biomining method that mobilizes the metals from the ores into the liquid medium. This happens as filamentous fungi excel at producing and excreting organic acids, performing an organic acid-mediated bioleaching. The fungus *Penicillium simplicissimum* is currently used to biomine copper and has been reported to effectively mine valuable

components from electronic waste[17]. *P. simplicissimum* bioleaches via the fungus' natural production of organic acids, such as citric or oxalic acid. These organic acids are secreted into the surrounding medium where they interact with the minerals, breaking down chemical bonds. As a direct result from the accumulation of the organic acids in solution, the pH lowers, which increases the solubility of most metals and promotes the release (or leaching) of metals into the leachate solution[14]. This process is called acidolysis, which is dominant in fungal bioleaching, although other biomechanical and chemical processes can simultaneously take place to help mobilize metals from the regolith being bioleached.

Given the wide use of filamentous fungi on Earth's industrial biotechnology (from antibiotic production, to food preservation or cosmetics) fungal biomining in space can be a promising ISRU technology, to provide the crew with metals for construction pipelines or energy generation, as well as to aid waste-management through fungal biodegradation and bioremediation[18–23]. From a logistical perspective, the implementation of fungal bioleaching can be easily carried to space, as fungal spores are dormant resistant structures and are known to be highly resistant to space radiation, making them favourable players for transport and stowage during space travel[18,24]. Besides, filamentous fungi *Aspergillus* spp. and *Penicillium* spp. are able to grow outside Earth's gravitational conditions, as they are commonly identified as part of the microbiome of space stations in Low Earth Orbit (LEO), such as Mir and the International Space Station (ISS)[18,24,25], and will likely continue to be present in future crewed long-term missions to the Moon.

A different gravitational regime on the Moon creates the necessity of fully comprehending the effect of gravity, not only on humans, but also on technological and microbial processes to be carried out *in situ*[26]. Space biomining has been successfully tested aboard the International Space Station, demonstrating the viability of using bacteria, such as *Bacillus subtilis* and *Sphingomonas desiccabilis*, to extract rare-earth elements[27]. *S. desiccabilis* showed no significant differences in metal mobilization in response to different gravitational regimes, however the biomining potential of *B. subtilis* was reduced. Recent developments in space biomining have been exclusive to bacterial species, however, based on what we know from fungal biotechnology on Earth, fungal biomining in space is likely a more promising line of research[28].

In this study we have taken the first steps to establish fungal biomining of lunar regolith. We selected the fungus *Penicillium simplicissimum* to develop a biomining setup that extracts metals from the Lunar regolith simulant EAC-1A. We report data on biocompatibility of *P. simplicissimum* to EAC-1A, under both Earth and simulated Lunar gravity. We demonstrate *P. simplicissimum's* ability to mobilize non-rare metallic elements from EAC-1A regolith into the medium solution (leachate). Additionally, we present data on fungal bioleaching culture profiling parameters (pH, total iron and organic acid levels), as well as quantification of bioleached metals by Inductively coupled plasma mass spectrometry (ICP-MS). Finally, we show the successful metal recovery from the leachate, and characterize the obtained powder via SEM/EDS (Energy Dispersive X-Ray Spectroscopy) and XRD (X-Ray Diffraction).

Results

Biomining of lunar regolith can provide future crews with useful metals produced in situ, in a cost-effective and sustainable way. In this study, we investigated biomining of EAC-1A Lunar regolith simulant using the filamentous fungus *P. simplicissimum*. We first assessed the fungus' tolerance to EAC-1A, in both Earth and Lunar simulated gravity conditions. We then performed bioleaching verification tests to evaluate the fungus's ability to extract non-rare metallic elements from the Lunar regolith into culture medium (leachate). Finally, we established a fungal bioleaching setup that enabled recovery of several metals in powder (Fig. 1).

Biocompatibility with EAC-1A lunar regolith simulant

Given the potential toxicity of the Lunar regolith and its simulants[29–31] we evaluated the biocompatibility of EAC-1A to *P. simplicissimum*, monitoring colony area under increasing concentrations of Lunar regolith of 0%, 0.5%, 10%, 20%, 40% and 60% (Fig. 2). Results show a decrease in colony area when the fungus was exposed to the highest concentration of EAC-1A (60%) at day 2 ($p < 0.001$) and day 4 ($p < 0.001$), suggesting a stress response of the fungus to the regolith simulant (Fig. 2a). However, by day 6, no difference was detected in fungal colony area when grown with lunar regolith ($p = 0.2$), suggesting an adaptation of the fungus to the supplementation of EAC-1A over time. A second run of biocompatibility ($n = 8$) was performed, comparing growth at 0% and 60% EAC-1A (w/v) over a longer incubation period of 9 days (Fig. 2b, 2c). Results show that when grown in 60% EAC-1A, the colony area of the fungus was significantly smaller by 35% at day 2, and smaller by 31% at day 4, ($p < 0.001$). However, on day 9, supplementation with EAC-1A 60% registered a 20% increase in colony area ($p < 0.001$), again suggesting an adaptation of the fungus to the supplementation of EAC-1A over time. Supplementation with 60% EAC-1A regolith also impacted colony morphology (Fig. 2c), with vegetative growth (white colour of the hyphae cells), and spore formation (green colour due to the spores' pigmented cell walls) differing in the presence of 60% EAC-1A regolith, when compared with growth without regolith (Fig. 2c). Overall, our finding suggested that exposure to Lunar regolith EAC-1A did not prevent or significantly impacted growth of *P. simplicissimum* from day 4 onwards.

Biocompatibility under Lunar Simulated Gravity (LSG)

To understand how Lunar gravity might impact fungal growth, *P. simplicissimum* was grown in PDA plates supplemented with 60% EAC-1A, or without EAC-1A (0%) that were exposed to Lunar Simulated Gravity (LSG) on a 2-D Petri-dish Clinostat set to a 10° angle (Fig. 3a). Changes in colony area (Fig. 3b), dried biomass (Fig. 3c), and spore production (Fig. 3d) were measured after 4 days, and compared to an Earth gravity control (EG). In colonies grown without EAC-1A (0%), exposure to Lunar Simulated Gravity (LSG) promoted a 20% increase in colony area ($p = 0.003$) but did not affect biomass ($p = 0.6$) or spore production ($p = 0.7$), when compared to ground control (EG). In contrast, in colonies grown with 60% Lunar regolith EAC-1A (60%), exposure to Lunar Simulated Gravity (LSG) did not significantly impact

colony area ($p = 0.062$) (Fig. 3b), total biomass ($p = 0.2$) (Fig. 3c), or spore production ($p = 0.06$) when compared to colonies grown in normal gravitational conditions (EG) (Fig. 3d). However, when in Lunar Simulated Gravity (LSG), colonies with EAC-1A (60%) exhibited 15% less colony area ($p = 0.008$) (Fig. 3b), 46% less biomass ($p < 0{,}001$) (Fig. 3c), and 21% less spore production ($p < 0.001$) than colonies without EAC-1A (0%) (Fig. 3d).

Bioleaching Verification Tests

To validate *P. simplicissimum*'s capacity to mobilize metals from the regolith to the liquid medium, bioleaching verification tests were performed in bioreactor flasks by growing fungal cultures in full PDB medium with 60% EAC-1A regolith for 2-weeks (Supplemental Table 2). Additionally, because both low nutrient availability and presence of toxic metals could trigger changes in the fungus's bioleaching potential[17,32], we investigated the impact of using low nutrient (LN) medium (20% PDB), and a reduced amount of EAC-1A regolith (30%). Culture profiling parameters were monitored to inform the bioleaching process, these were: medium pH, total iron (Fe^{3+} and Fe^{2+}) concentration, and levels of organic acid (citric and oxalic acid) (Fig. 4 and Supplemental Tables 3–4).

Results show that fungal cultures in full medium without regolith (PDB REG-) reported an acidic pH 4, which remained acidic after 1 week of incubation, registering the lowest pH of 3.5 on day 5 (Fig. 4a). In contrast, the pH remained slightly alkaline at 7.5 in full medium cultures with EAC-1A regolith (PDB REG + and PDB REG++) where the fungal bioleaching process took place (Fig. 4a). Similarly, in bioleaching cultures using a low nutrient medium and a reduced amount of EAC-1A (LN PDB REG+), the pH profile of the medium was rather neutral to alkaline (pH 6–7.5) after 1 week of bioleaching, in contrast to the acidic pH 4 of low nutrient medium without EAC-1A (LN PDB REG-) (Fig. 4a).

Total iron was measured as an indicator of successful metal extraction from the regolith to the leachate solution. Results show that, in fungal cultures without regolith supplementation (REG-), the iron levels in solution remained at 0. In contrast, fungal bioleaching conditions (REG + and REG++) described mobilization of 20 mg/L to 50 mg/L iron from the regolith to the liquid environment by day 6. Additionally, data suggests that reducing nutrients improves bioleaching efficiency, as the experimental conditions using low nutrient medium (LN PDB) reached higher concentrations for iron (50 mg/L) than those using full medium. Furthermore, results show that using a lower volume of EAC-1A (30%) does not impact the final amount of iron being mobilized, when compared with a higher volume of EAC-1A (60%) (Fig. 4b and Supplemental Table 4).

Given the key-role of organic acids in the bioleaching process we measured the concentration of both citric and oxalic acid in the bioleaching cultures over 1 week. (Fig. 4c-d). Results indicate that both regolith supplementation and low nutrient medium (LN) induce changes in the levels of organic acids. From all tested conditions, the maximum levels of organic acids were achieved in cultures supplemented with Lunar regolith simulant EAC-1A during the first 4 days (intended biomining scenario). In fungal bioleaching cultures using full medium with 30% EAC-1A (PDB REG+), citric acid levels reached a

maximum concentration of 140 ± 29 mg/L, which was much higher than the levels achieved in cultures without regolith (91 ± 23 mg/L) (Fig. 4a). Cultures using low nutrient medium accumulated less citric acid than full-medium cultures, registering a maximum of 68 ± 7 mg/L when supplemented with 30% EAC-1A (at day 3), and 85 ± 4 mg/L in cultures without regolith (at day 2) (Fig. 4a). In contrast, the maximum levels of oxalic acid in the leachate (0.025 ± 0.015 mg/L) were achieved on day 2 in bioleaching cultures using low-nutrient medium with 30% EAC-1A (LN PDB REG+). In full medium with 30% EAC-1A (PDB REG+), the peak of oxalic acid occurred only on day 3 (0.008 ± 0.006 mg/L) (Fig. 4d), whereas in fungal cultures without EAC-1A supplementation, the maximum concentration of oxalic acid occurred only at day 7, in both low nutrient medium (0.016 ± 0.002 mg/L), and in full medium (0.022 ± 0.003 mg/L) (Fig. 4d).

Fungal Bioleaching setup

Bioleaching tests demonstrated that a lower nutrient medium and a reduced amount of EAC-1A regolith would maximize the metals mobilized while reducing the required resources. Therefore, a fungal bioleaching set-up was established using LN medium (20% PDB) supplemented with 30% EAC-1A (w/v). *P. simplicissimum* bioleaching cultures were grown in bioreactor flasks for two weeks, after which the leachate solutions were collected and subjected to analysis using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to identify and quantify the extracted metals (Fig. 5).

ICP-MS analysis demonstrated the capability of *P. simplicissimum* to extract different metals from EAC-1A, Results report an efficient extraction of metals during fungal bioleaching, of up to 159 mg/L magnesium, 151 mg/L calcium, 68 mg/L iron, 32 mg/L aluminium, 3 mg/L manganese as well as traces of titanium (0.02 mg/L) (Fig. 5). For comparison, a chemical leaching control (medium + organic acids) was included, which demonstrated successful metal mobilization, however, in much lower amounts than fungal bioleaching cultures. This demonstrates the significance of organic acids, particularly citric and oxalic acid, in mobilizing metals from the regolith particles into the leachate, and highlights the use of fungi to maximize the leaching process.

Interestingly, results indicate a baseline magnesium mobilization when using PDB medium (potato-based) for bioleaching, as an initial magnesium concentration of 4 mg/L in medium without regolith increased to 10 mg/L when supplemented with 30% EAC-1A and incubated for 2 weeks (Supplemental Table 5). It is important to note that PDB-led metal mobilization was not detected for any of the other metals analysed in this study. Results also display the fungus uptake of metal elements, as the concentration of metals dropped after 2 weeks in cultures with *P. simplicissimum* (but without regolith). This happened in particular with magnesium which concentration in the medium was 30% less (3 mg/L) than in flaks containing only PDB (without fungus).

Retrieval of metals from the leachate was successfully achieved, allowing for an extraction of 10.3 ± 3.7 g/L of a metal powder of mostly white color with specs of brown-coloured particles (Fig. 6a). Analysis of the obtained powder via Scanning Electron Microscopy (SEM) shows that heterogeneous particles that

differ in size, and subsequent Energy Dispersive X-Ray Spectroscopy analysis (EDS) analysis confirms the presence of aluminum, calcium, magnesium and iron, which validates the recovery process. Interestingly, EDS-analyzed powder fractions show a high abundance of aluminium, with a parallel secondary dominance in either sodium, iron, calcium, or magnesium (Fig. 6.d and Supplemental Fig. 1). This contrasts with the ICP-MS analysis which detected magnesium as the most abundant metal in the leachate solution. Further X-Ray Diffraction (XRD) analysis of the powder (Fig. 6b) reveals a spectrum mostly related to Boehmite (AlO(OH)), with lower amounts of Calcium Carbonate ($CaCO_3$) and Halite (NaCl) adding to the broadened aluminium-related peaks. It should be noted that the minerals periclase (MgO) and haematite (Fe_2O_3) might also be present in the samples, however signal overlap of the observed peaks could not confirm their detection. Other metals were present in the powder sample, for instance, palladium (Fig. 6d) which signal presence can be traced back to the sputter layer which was applied during sample preparation of SEM/EDS analysis, as well as sodium and chloride, which signals likely result from the precipitation method used to recover the metals.

Discussion

To become sustainable and cost-effective future missions to the Moon will depend on IRSU technologies such as mining of the Lunar soil[5,7]. Indeed, the Lunar regolith contains minerals with high levels of metallic elements, which are often toxic to microorganisms[29]. Nonetheless, filamentous fungi can tolerate high concentrations of metals, making them suitable candidates for biomining applications[33,34,35].

Therefore, in this study, we investigated the ability of the filamentous fungus *P. simplicissimum* to extract metals from the Lunar regolith simulant EAC-1A. We envisioned a Moon biomining scenario which takes place inside a habitat exposed to Lunar gravity (0.16 g), shielded from radiation, and with an optimal temperature for its occupants (of 22°C, similarly to the ISS). Our initial tests demonstrate that the fungus *P. simplicissimum* is biocompatible with EAC-1A, even at high concentrations of up to 60% (Fig. 2) and that Lunar Simulated Gravity (0.16 g) on a 2-D Petri-dish clinostat did not significantly alter *P. simplicissimum* growth (Fig. 3). Given the fungal biocompatibility, our bioleaching tests report the ability of *P. simplicissimum* to efficiently mobilize iron (up to 50 mg/ml) using low nutrient medium with a reduced amount of EAC-1A regolith. This is particularly important as the medium chosen is based on potato infusions and sugar (which is cost-effective, non-toxic, and can possibly be obtained from potatoes grown on board) allowing for future fungal bioleaching approaches to minimize the use of resources. We furthermore report a successful fungal bioleaching of EAC-1A regolith by the fungus *P. simplicissimum*, using 40 mL bioreactor flasks with low nutrient medium (20% PDB) and 30% EAC-1A (w/v), which results in the extraction of magnesium (159 mg/L), calcium (151 mg/L), iron (68 mg/L), aluminium (32 mg/L), manganese (3 mg/L) and titanium (0.02 mg/L) in the leachate solution, after 2 weeks at room temperature (22°C). Moreover, we report the successful metal recovery from the leachate solution to a promising average of 10 ± 3 g/L of metal powder. Further SEM/EDS and XRD analysis confirm the high abundance of aluminium [as boehmite (AlO(OH))], magnesium [possibly as periclase

(MgO)], and iron [possibly as haematite [possibly as (Fe_2O_3)] and magnetite [possibly as (Fe_3O_4)] in the recovered metal powder. The minerals extracted can be further purified and refined to be used in technology production for construction materials or power generation devices[7,12]. For instance, the extracted magnesium (159 mg/L) is a metal whose alloys are known for their light structure and is therefore highly attractive for space exploration, where increased weight of equipment always requires the use of more fuel[36,37]. In turn, usage of an *in situ*-produced aluminium could be included in the manufacture of space vehicles, and solar cells[38,39]. Moreover, novel research has shown that biomined iron has potential for use in synthesizing components (replacement parts such as screws) that could integrate systems for space exploration[40]. Therefore, metals extracted via fungal bioleaching can aid future Lunar habitats in becoming independent from Earth, and possibly create avenues for commercial contributions[4].

Biomining of lunar regolith simulant has also been demonstrated by Kaksonen *et al.* using acidophilic bacteria (*Acidithiobacillus ferrooxidans*), resulting in successful mobilization of metals such as magnesium (~ 300 mg/L) and aluminium (~ 60 mg/L), after 20 days incubation. However, the study used a different regolith simulant – 1% Lunar Mare Simulant (LMS-1) – which was supplemented with 12.5 mM Fe[3,41], whereas the current study used EAC-1A lunar regolith simulant, which more similar to the Lunar regolith obtained from Apollo 17 samples[9]. Interestingly, the driving biomining mechanism described for *A. ferrooxidans* was acidolysis – the low pH of the cultures leading to the mobilization of the metals into solution. In contrast, in our study fungal bioleaching of EAC-1A seemed to have occurred mainly at a slightly alkaline pH (7.5). Besides, our results show that the peak accumulation of organic acids in the leachate did not correlate with the minimum pH measurements, or with the maximum amount of metals mobilized. These findings challenge the assumption that acidolysis is the main driving mechanism for fungal bioleaching of EAC-1A regolith[42]. In turn, we suggest complexolysis to be one of the main processes in EAC-1A bioleaching by *P. simplicissimum*. During complexolysis, organic acids react with the metals, forming either soluble or precipitated complexes that help stabilize the metal ions from the regolith[43,44], reducing their bioavailability and therefore also reducing metal toxicity toward the fungus[45]. This is further supported by XRD analysis of the final metal powder obtained, where a broad peak for boehmite, a mineral containing aluminium, suggests a reaction of the metal with secondary metabolites, which may include organic acids[46]. Metal oxalates, such as calcium oxalate, are formed during fungal bioleaching of EAC-1A, and have low solubility at alkaline pH, which leads to the precipitation of the chelated metals[47,48]. Other bioleaching processes might also be involved in fungal bioleaching, although certainly at a smaller scale. For instance, redoxolysis, where enzymatic reactions modify the oxidation state of the metal, enhancing its solubility[49] and bioaccumulation (where metal ions can be assimilated into living cells, either in vacuoles or the cell wall[50]). Moreover, from a biological point of view, it is also important to note that a filamentous fungus, such as *P. simplicissimum*, have a vast metabolome. This indicates that *P. simplicissimum* has the capacity to produce various other organic acids (such as gluconic acid) and an array of other metabolites[51]. Such compounds may have an impact on the bioleaching potential, especially those with chemical groups such as the carbonyl, amino, hydroxyl

and nitro groups, which can interact with the metals on the regolith[52,53]. Moreover, the size of the regolith particles can trigger changes in bioleaching, possibly leading to differential metal extraction[54]. For example, industrial applications often require smaller pellets due to rheological (outflow) problems[55].

Although our study demonstrates fungal bioleaching as promising ISRU technology for future Moon missions, real application of fungal biomining in space will require a deeper understanding of the bioleaching mechanisms, as well as further optimization and modifications of the process to accommodate both downscaling, for miniaturized testing at Low Earth Orbit (e.g. aboard the ISS), as well as upscaling, for high-volume testing of technologies (e.g. ground-based facilities such as the ESA-DLR LUNA habitat)[12]. Another critical aspect would be to develop a medium with fewer nutrients, and fewer lunar regolith, without compromising the metal mobilization of the tested fungal bioleaching setup. Small-scale biomining has been recently demonstrated on board the ISS with bacteria extracting rare earth elements under different gravitational regimes, and from different regolith analogues[27,56]. Additionally, a previous study employed a novel miniaturized space biomining reactor to conduct metal leaching with bacterial strains *S. desiccabilis, B. subtilis* and *Cupriavidus metallidurans*. Apart from the upcoming Bioasteroid mission aboard the ISS, that will test filamentous fungi potential to mobilize metal onboard the ISS[14], to our knowledge there are no studies addressing fungal biomining in a space-relevant scenario, highlighting the importance of our study.

We therefore emphasize the use of fungal biomining in space exploration scenario, as it offers several advantages. It is lightweight[24]; non-exclusive, allowing the selective precipitation of specific metals according to the crew's necessities[14,57]; and can be paired with other processes within the space habitat, for example, with bioremediation of soil for agriculture[58], or electronic waste-recycling[51,59,60]. Besides, the versatility of biomining as ISRU means it can be applied further beyond the Lunar regolith[14,27,61]. Similarly, we envision the utilization of fungal biotechnology as an crucial tool to achieve sustainability and circular economy, both on Earth and in space[62].

Methods

Media and Strain

In this study, the filamentous fungus strain used was *Penicillium simplicissimum* (DSM 1097) obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The fungal spores were harvested from three to four days-old cultures, grown on Potato Dextrose Agar (PDA) (Sigma), at room temperature (22°C). Spore harvesting was performed by placing 5–10 mL of saline solution (0.9% NaCl) on the agar plate and gently scraping the surface using a cotton stick. The spore suspensions were retrieved from the agar plate and then filtered with a Miracloth filter (Millipore) and stored at 4°C. Spore concentration was determined using a Thoma counting chamber. All experiments were performed with fresh spore suspensions not older than 2 weeks. The Lunar regolith simulant used (EAC-1A) was provided by Dr. Aidan Cowley from ESA Spaceship EAC, Cologne, Germany. Composition of EAC-1A regolith is

described by Engelschiøn *et al.* 2020. In all experiments utilizing medium supplemented with regolith, the regolith was previously sterilized in a powder form inside a glass petri dish or shake-flasks in a heat steriliser at 220°C for 4 hours.

Biocompatibility Tests

To test biocompatibility of the fungus with the regolith, the fungus was growth on PDA plates supplemented with different concentrations of EAC-1A [0%, 0.5%, 10%, 20%, 40% and 60% (w/v)]. Agar-medium plates supplemented with EAC-1A can be challenging due to insolubility of the regolith. Thus, to obtain EAC-1A agar plates, this study used the following method: The Lunar regolith EAC-1A powder (previously heat sterilized) was first weighted to the desired amount (to each corresponding EAC-1A concentration) using a precision scale and then transferred to a media bottle (Schott) to which was added PDA in accordance with the manufacture' instructions. After autoclaving, the mixture (EAC-1A and agar) was agitated, and 30 mL were collected from with a 50 mL disposable sterile pipette. Due to the low solubility of EAC-1A, the volume was collected from the interface to an empty, and sterile petri-dish to achieve plates with uniform regolith concentration. To test fungal growth, the plates were inoculated with 10 µL of a spore suspension with 10^6 spores/mL for 6 days at room temperature (22°C). Fungal growth was monitored via the determination of the corresponding colony area on days four and six. A second biocompatibility assay was performed with the same methodology, comparing fungal colony area at 0% and 60% EAC-1A (w/v), from zero to nine days, with measurements on days two, four, seven and nine. Determination of colony area was calculated from high resolution photographs taken with the camera Sony α-500 (APS-C), with a macro-objective (E 3.5/30). A tripod was used to ensure a constant height. Photographs taken were analysed with the Fiji/Image J software, where the colony area was measured[63].

Growth in Lunar Simulated Gravity (LSG)

To study the adaptation of the fungus *P. simplicissimum* to Lunar Simulated Gravity (LSG), agar plates with normal medium (PDA) and PDA supplemented with 60% EAC-1A were incubated on a 2-D petri dish clinostat. The simulation of Lunar gravity is achieved by the principle of clinorotation in which samples are continuously rotated, at a constant speed, and are thus exposed to a functional simulation of an altered gravity environment at the centre of the rotation axis[64]. It is important to note that the clinostat provides a functional simulation that prevents particle sedimentation. When placing the colony at the centre of the plate, the simulated Lunar gravity can be calculated with the following equations:

$$a = \omega^2 r$$

$$\omega = 2\pi\ rpm60^{-1}$$

Where *a* represents the centripetal acceleration (m s^{-2}), *ω* represents the angular velocity (rad $second^{-1}$) and *r* represents the colony radius. The degree to the horizontal axis of the clinostat was calculated

accordingly, which suggests a 10° angle of the rotation axis, to achieve 0.16 g, this is Lunar Simulated Gravity (LSG) (Fig. 6). Plates of the experimental LSG group were placed in the clinostat ($n = 3$), and ground control Earth gravity (1g), by plates were placed on the bench ($n = 3$). Both groups were incubated at room temperature (22°C) for four days. Adaptation of the fungus to LSG was monitored after four days via measurements of colony area (see section above), dry biomass and spore production. Dry biomass was determined by preparing PDA plates supplemented with 0% and 60% EAC-1A and placing an additional removable polycarbonate-filter (0.4 µm pore size) on top of the agar. This was followed by inoculation with 10 µL of fungal spore suspension, at a concentration of 10^6 spores/mL, after which the plates were incubated at 22°C. At the end of the fourth day, the filter was removed, detaching the colony from the PDA plate. Each filter carrying a colony was placed inside of a pre-weighted aluminium paper. The weight of the aluminium foil with the colony was measured before and after desiccation (dw) at 60°C for 24 h, with a high precision analytical scale (Sartorius). Dried Biomass was calculated in accordance with the following equation:

Colony Biomass = Dry weight (dw) − Filter's weight − Aluminium paper's weight.

Spore production was determined according to the protocol described in Cortesao *et al* 2022[65].

Bioleaching Verification Tests

To test relevant parameters that would inform the final setup: nutrient concentration, w/v of EAC-1A, temperature of incubation, bioreactor flasks were used, each with 40 mL volume of 100% or diluted 20% Potato Dextrose Broth (PDB) medium, to test the effect of low nutrient medium in bioleaching ability. The medium was supplemented with 0%, 30% and 60% (w/v) concentrations of Lunar regolith simulant EAC-1A. To prepare the bioleaching cultures, the first step was to add 12–24 g the powder EAC-1A to the bioreactor flasks, which were then submitted to sterilization in the heat-sterilizer (220°C for four hours). After which sterile medium, and fungal spore suspensions were added. In total, each flask was inoculated with 2.5 x 10^5 spores (final concentration) and was incubated under agitation at 150 rpm and 22°C for two weeks. The tested bioleaching cultures were monitored in the first week, by measuring medium parameters via semi-quantitative methods, according to the manufacturer's instructions: pH (strips, Sigma), organic acids (citric and oxalic acid kits, Sigma), and metal concentration: iron, aluminium, and calcium (Kits, Sigma).

Fungal bioleaching setup

Erlenmeyer flasks with 40 mL total volume of PDB diluted for 20% of total nutrients or non-diluted PDB (100%), each supplemented with either 0% or 30% EAC-1A were prepared. The bioreactor flasks were inoculated with 2.5 x 10^5 spores and kept under agitation at 150 rpm. The final bioleaching setup is schematically represented in Fig. 1. A chemical leaching control group (not inoculated) was designed for the maximum concentrations of citric acid (0.15 g/L) and oxalic acid (0.008 g/L) measured at the end of

two weeks on a bioreactor flask supplemented with EAC-1A. A non-inoculated group of bioreactor flasks with diluted PDB (20%) with 30% EAC-1A (w/v), and diluted PDB (at 20%, and with 0% EAC-1A) were used as blanks for comparison. The bioleaching process was monitored via measurement of organic acid production: citric acid (Kit, Megazyme) and oxalic acid (Kit, Sigma-Aldrich) levels were measured according to the manufacturer's instructions over the first week (on days two, three, four and seven). By the end of two weeks, the biomass and the regolith were removed by centrifugation at 4000 rpm for 30 minutes to isolate the leachate. The samples were then sterilized by filtration with a Whatman paper filter of pore size 0.2 µm before adding the samples, 15 mL falcon tubes were washed with MilliQ water to prevent chemical contamination. The solutions were kept at 4°C until shipped for precise detection and quantification of the metal ions by ICP-MS (service of Medizinisches Labor, Bremen, Germany).

Metal recovery and spectroscopy/phase analysis

Recovery of the metals from the leachate was performed by selective chemical precipitation ($n = 3$). This process precipitates the metals by adjusting the pH progressively to 10 with NaOH and HCl (both elements detected in the XRD results). Separation of the metals of interest from the leachate was done with cycles of centrifugation at 14 500 *g* (4000 rpm) for 20 minutes, due to their partial solubilities. The collected metals were then dried at 60°C overnight, resulting in a final precipitate's powder. The metallic nature of the resulting powder was initially confirmed by re-solubilization and measurement by the semi-quantitative methods described. Analysis of the powder content provided information on the chemical nature of the recovered metals. Precipitate's analysis included Scanning Electron Microscopy and Energy Dispersive X-Ray Spectroscopy (SEM/EDS) to validate the metallic content of the samples and XRD to confirm spectroscopic analysis and detect relevant crystallographic phases. SEM was conducted on a dualbeam workstation (*Helios Nanolab 600* by *FEI*) including the system´s EDS module. SEM imaging and EDS was conducted in secondary electron (SE) contrast mode at an acceleration voltage of 15 kV and a current of 1.4 nA. To allow for analyzation, the powder samples were collected on adhesive carbon patches, while a ~ 10 nm layer of Pd was applied via sputter coating prior to analysis to improve sample conductivity. X-Ray Diffraction (XRD) was performed on a *PANalytical Empyrean* diffractometer using Bragg-Brentano geometry and a low background silicon single crystal sample holder plate. On primary side a copper tube and low background optics (BBHD) and fixed slits were used, whereas the secondary beam path contained a fixed anti-scatter slit with semiconductor detector PIXcel3d in 1D channel mode.

Statistical analysis

Data analysis and graphical representation were performed using SigmaPlot 14.5 software. All experiments were conducted with at least $n = 3$ replicates, and the graphs displayed here show the mean and standard error. Statistical analysis was performed using the Kruskal-Wallis test for non-parametric datasets (biocompatibility – 9 days) and Analysis of Variance (ANOVA) for parametric datasets (biocompatibility – 6 days), after passing the Shapiro-Wilk (normality test) and Brown-Forsythe (equal variance test). In all cases, statistical significance was considered when p-value < 0.05. The statistical differences in biocompatibility under Lunar Simulated Gravity (LSG) were determined using ANOVA for

the parametric dataset (colony area, biomass, and spore production), which also passed the Shapiro-Wilk (normality test) and Brown-Forsythe (equal variance test).

Declarations

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Author contributions

J.F. and M.C. designed the study and interpreted the data. J.F performed the experiments and analysed the data. D.W.M. and S.W. executed SEM/EDS and XRD analysis and interpretation. J.F and M.C. co-wrote the manuscript which was approved by all co-authors.

Data availability statement

All data generated during this study are included in this published article and its supplementary information files. No additional data were generated or analysed.

Additional Information

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The authors declare no competing interests.

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Figures

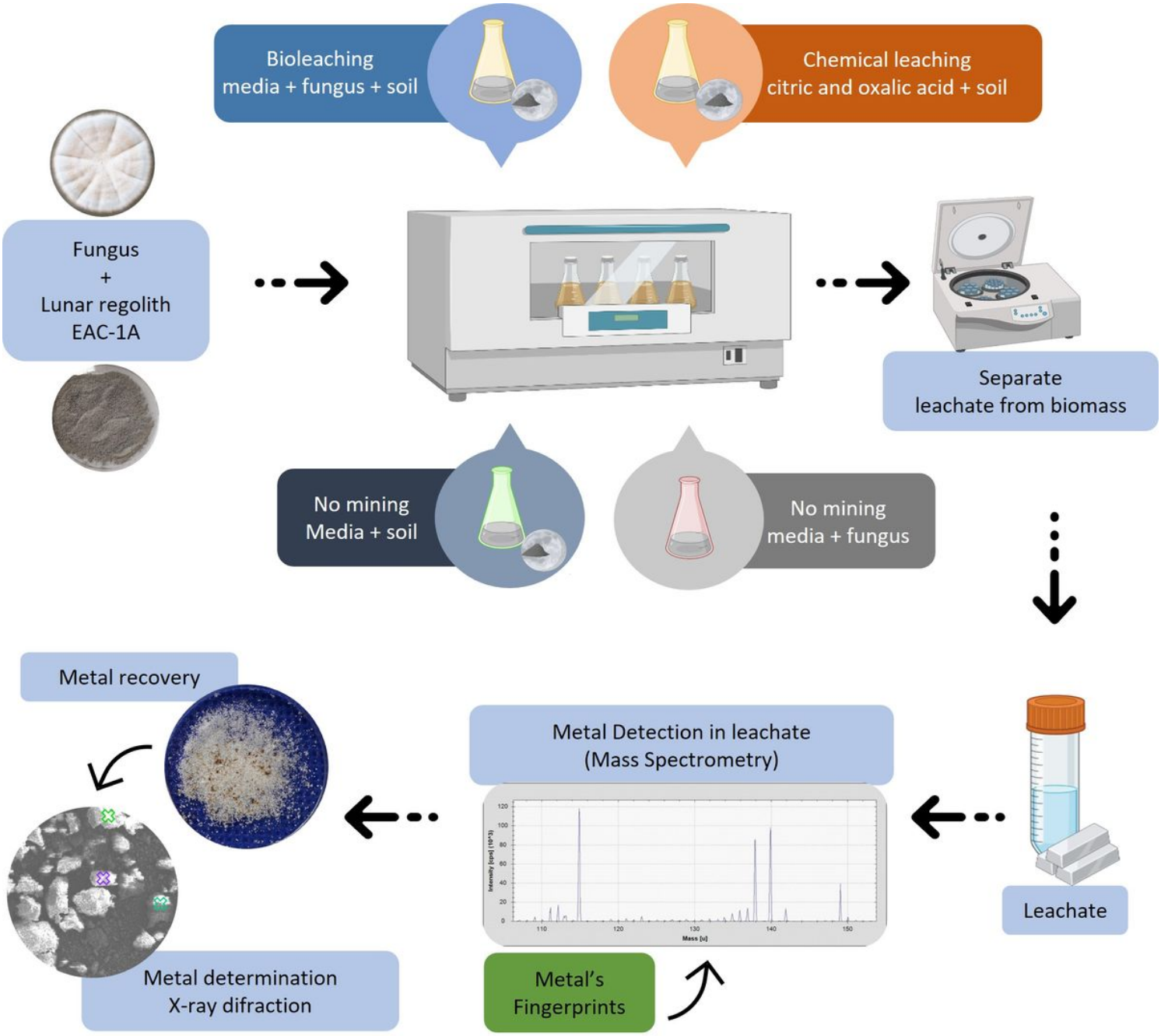

Figure 1

Schematic representation of the fungal bioleaching setup and procedure for metal quantification. After 2 weeks of bioleaching the liquid fraction of the cultures (leachate) was collected and sterilized for a sensitive quantification of the metal ions in solution by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

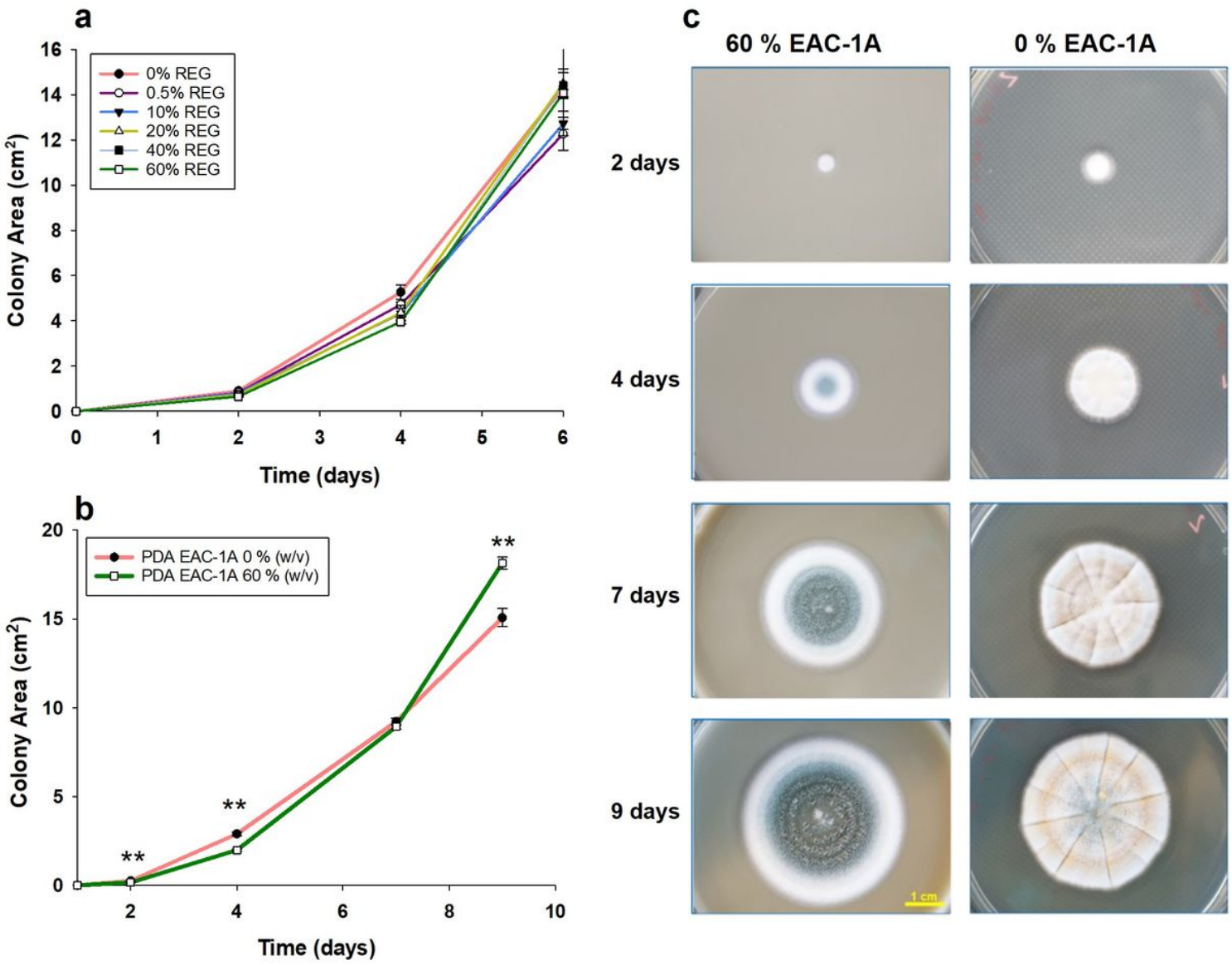

Figure 2

Biocompatibility of the fungus *P. simplicissimum* to EAC-1A lunar regolith simulant.
(a) Colony area of fungus grown in PDA medium supplemented with 0 %, 0.5 %, 10 %, 20 %, 40 % and 60 % (w/v) of EAC-1A regolith, over 6 days (n = 3 per group). (b) Comparison of fungal colony area in 0 % and 60 % EAC-1A over 9 days (n = 8 per group). (c) Colony morphology of *P. simplicissimum* grown in PDA medium with 60 % EAC-1A regolith (left), and without EAC-1A (right). Yellow bar corresponds to 1 cm and is valid for all images. ** represents $p < 0.001$ (Kruskal-Wallis test).

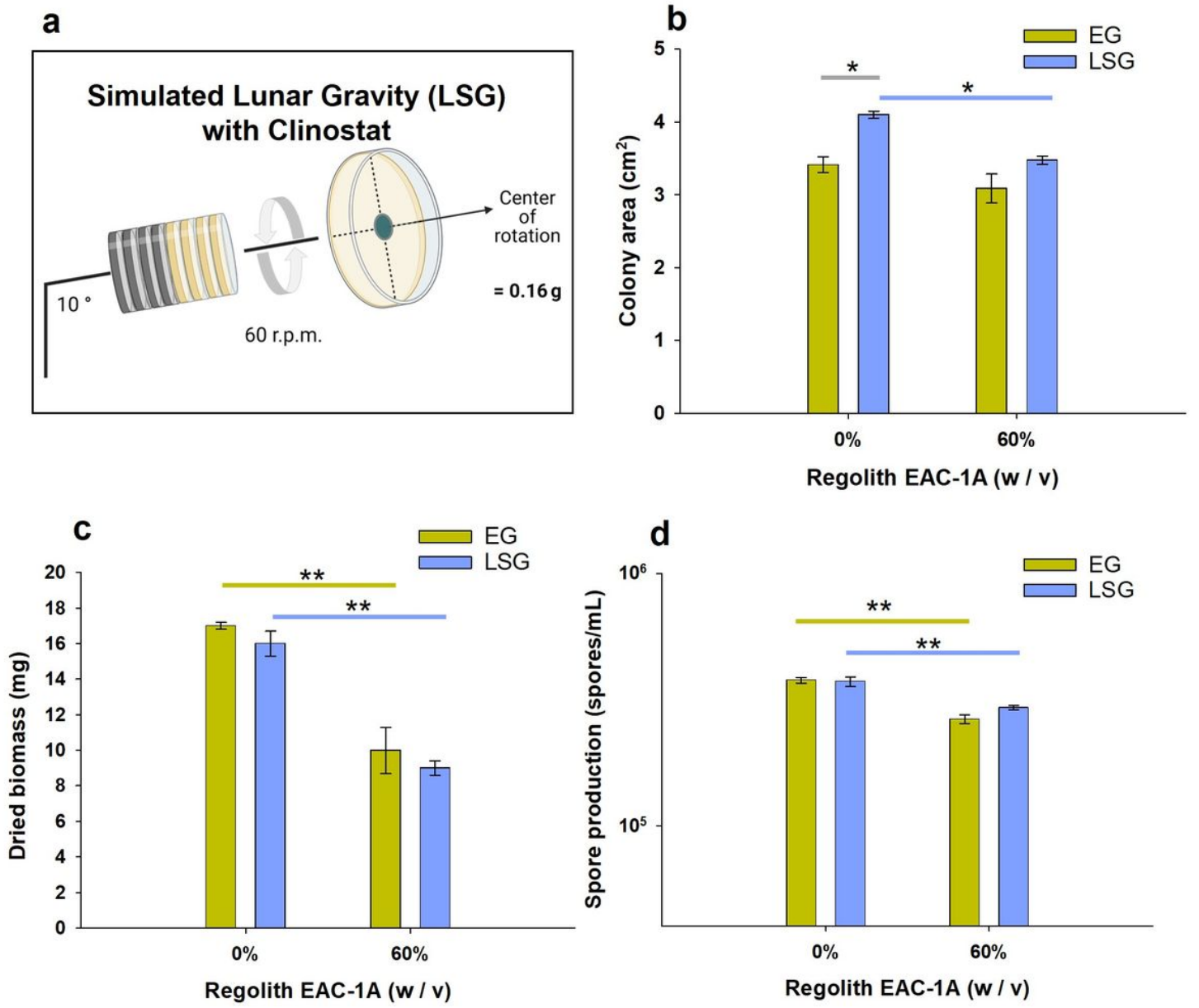

Figure 3

Impact of simulated Lunar gravity (LSG) on *P. simplicissimum* growth parameters when grown in agar plates with and without EAC-1A, over 4 days. (a) Set-up of 2-D petri dish clinostat with rotation axis set at 10° angle to simulate Lunar Gravity (LSG).(b) Colony area of the fungus in exposure to EG and LSG, when supplemented with 0 or 60 % EAC-1A, suggesting a slight increase of the colony area when exposed to LGS and a significant decrease on the colony area when supplemented with EAC-1A under Earth's gravity (n = 3 per group). (c) Dried biomass of the fungus in exposure to EG and LSG, when supplemented with EAC-1A suggesting a decrease caused by the supplementation of the medium with EAC-1A in both gravitational regimes (n = 3 per group). (d) Spore production in exposure to EG and LSG, when

supplemented with 60 % EAC-1A suggesting regolith leads to less spore production both EG and LSG (n = 3 per group). Where * represents $p < 0.05$, and ** $p < 0.001$ (One Way ANOVA)

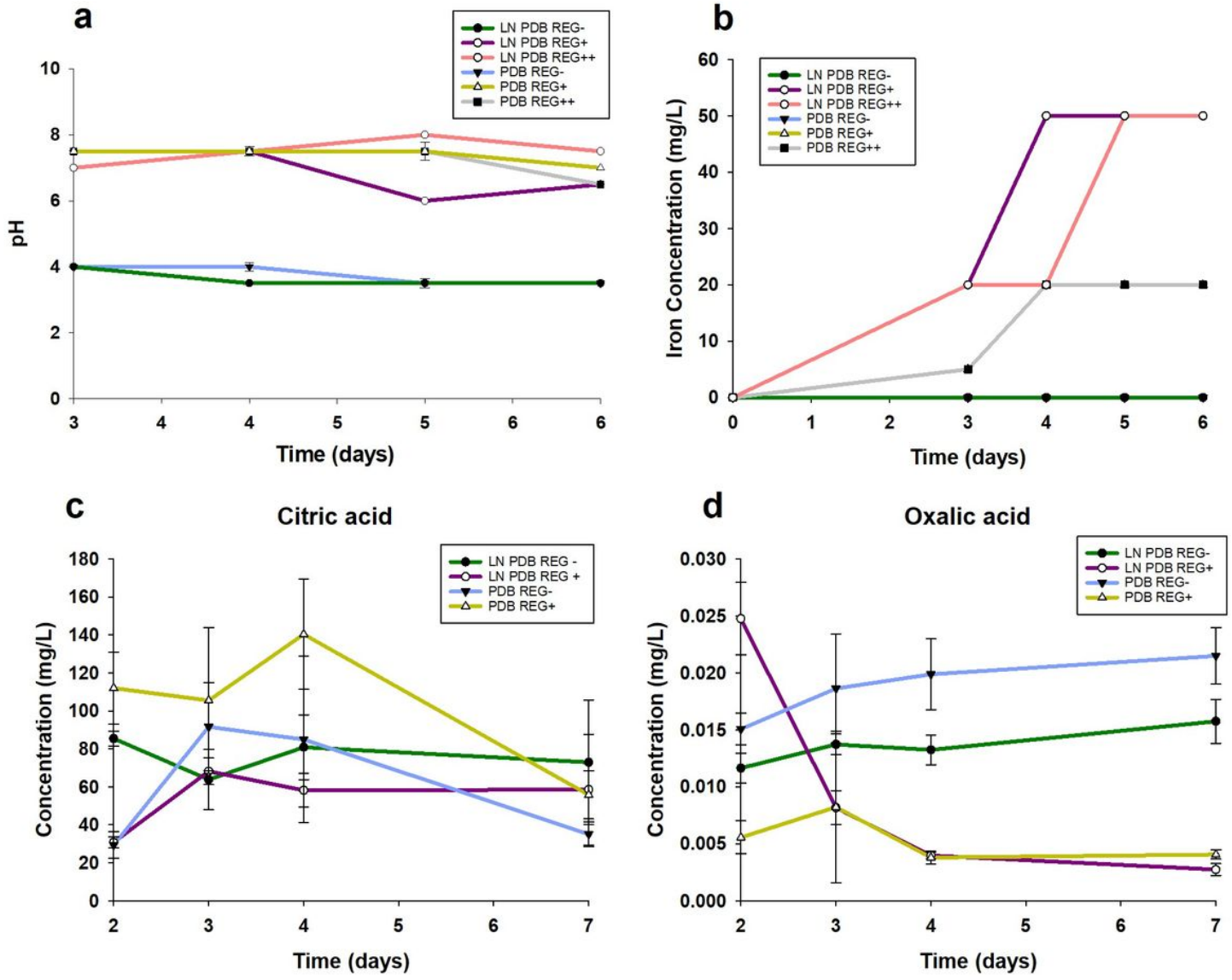

Figure 4

P. simplicissimum bioleaching cultures profiling parameters in 20 % PDB (v/v) over 1 week (n = 3). (a) pH variation on the leachate. (b) Iron ions concentration on the leachate. (c) Citric acid levels suggesting an initial peak between day 3 and day 4, and the maximum value recorded for the cultures of PDB with 30 % EAC-1A (REG+) (sd = 0). (d) Oxalic acid levels suggesting an initial peak for cultures supplemented with 30% EAC-1A (REG+) after which the concentration lowers over time. REG+ represents supplementation of the media with EAC-1A at 30 % (w/v).REG++ represents the supplementation of the media with EAC-1A at 60 % (w/v).

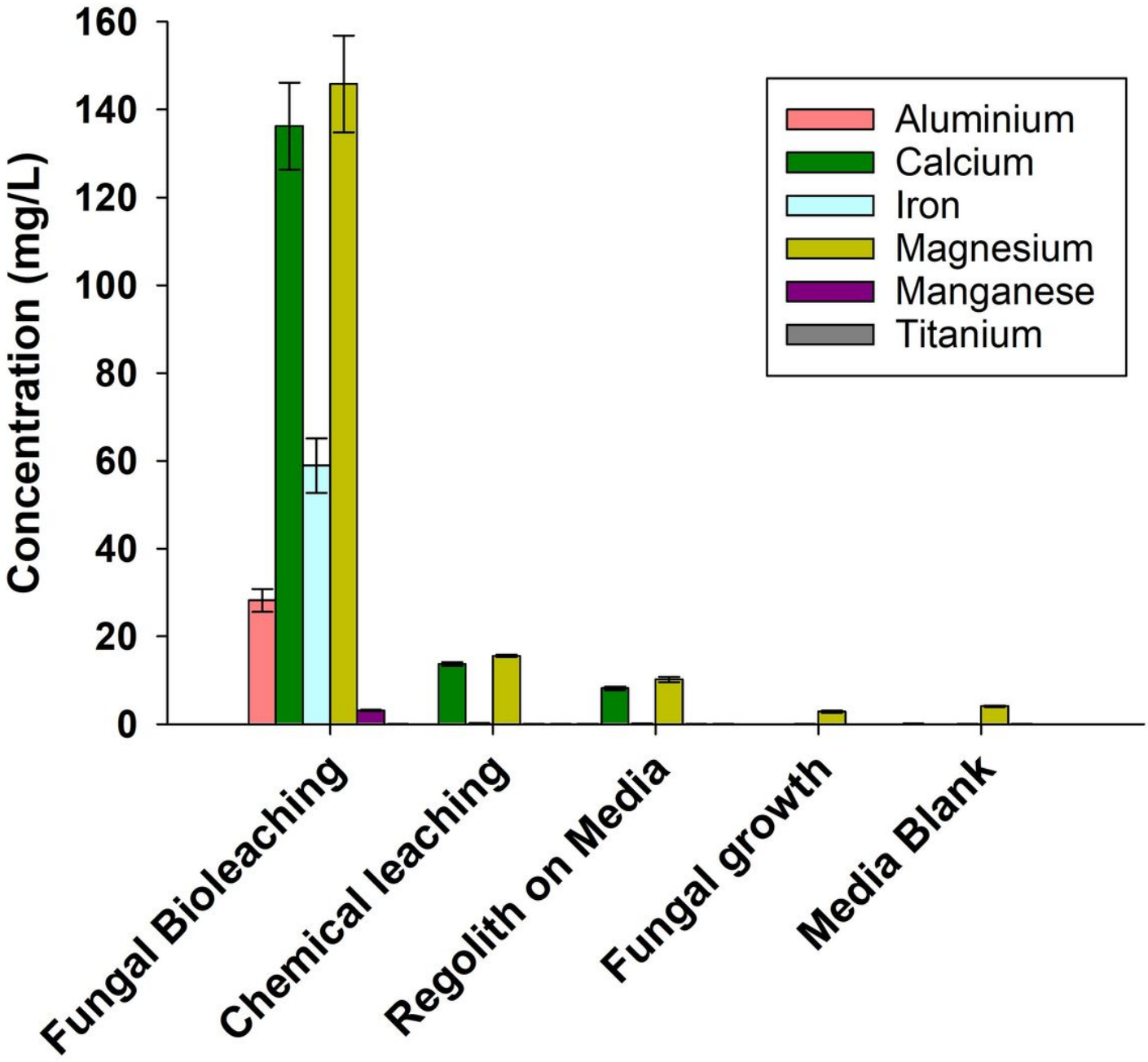

Figure 5

Metallic ion concentrations (mg/L) in the leachate solutions after 2 weeks of fungal bioleaching process using LN media with 30 % EAC-1A (w/v), demonstrating the viability of using the fungus *P. simplicissimum* to extract the metals from Lunar regolith *(n* = 3 per group).

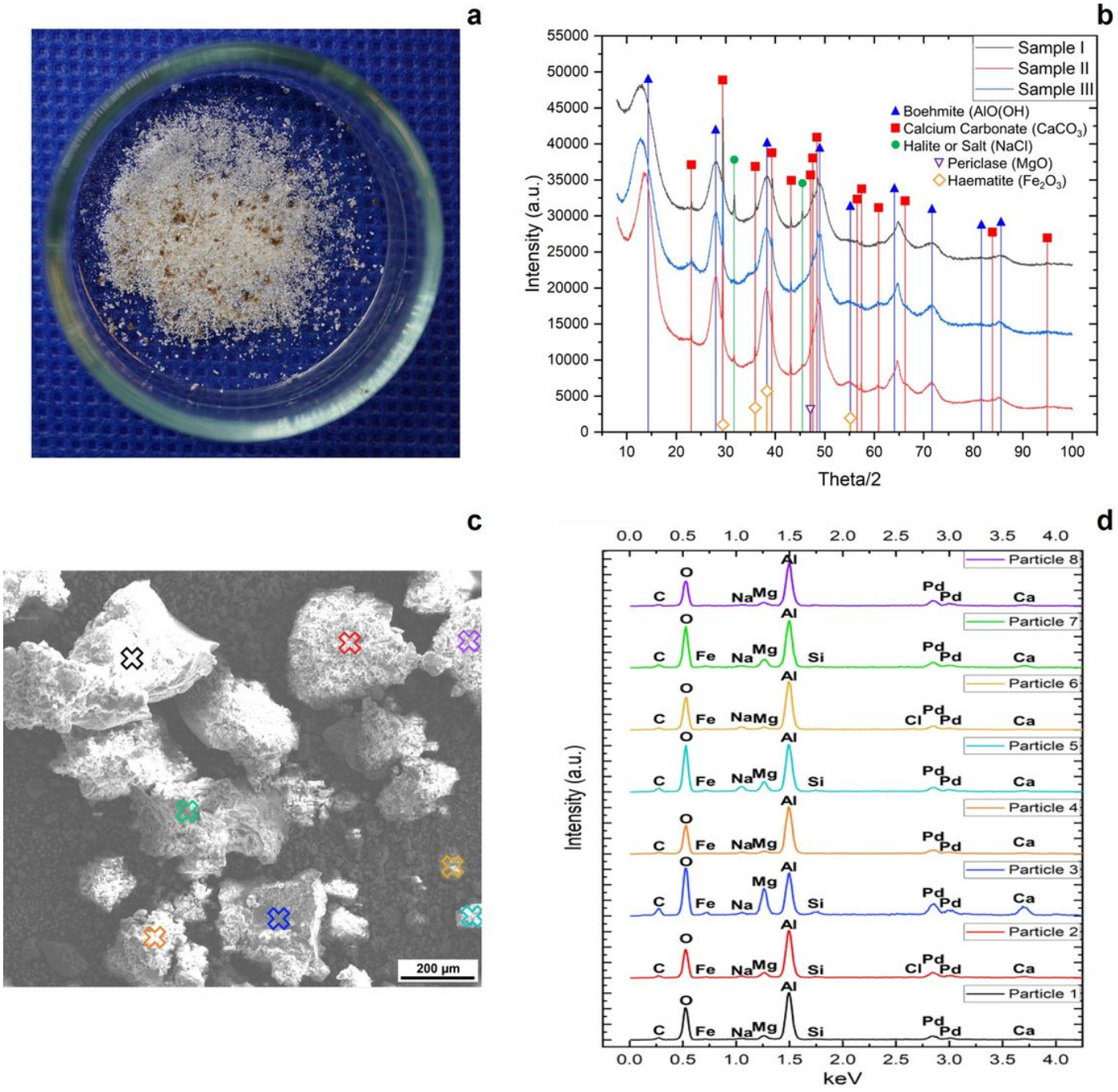

Figure 6

Metals recovered by chemical precipitation from the leachate solutions of the fungal bioleaching cultures of EAC-1A (*n* = 3). (a) Macroscopic view over the mineral powder (b) Energy Dispersive X-Ray Spectroscopy analysis of the powder evidencing the presence of metals successfully recovered from the leachate (aluminum, calcium, magnesium and iron) (c) Scanning Electron Microscopy of a powder sample, showing heterogeneous particles that differ in size (d) X-Ray Diffraction of the mineral powder, detecting metals successfully recovered from the leachate (aluminum, calcium, magnesium and iron).

Supplementary Files

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