Aluminum extraction through biomining using *Aspergillus foetidus* and future implications for in-situ resource utilization

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The moon offers mineral resources such as aluminum. Biomining, the process of using microorganisms to extract metals of economic interest from rocks and regolith, offers an attractive method for making in-situ resource utilization of lunar minerals possible. Organic acids, integral to fungal metabolism, are utilized in the biomining process for leaching metals. Biomining is currently being used on earth, in the mining industry to extract Cu, Al, Fe, and Au and obviate the requirement for toxic chemicals.

Nomenclature

Al	= Aluminum
ELGA	= Water purification system employing reverse osmosis, microfiltration, ultrafiltration, degassing, ultraviolet photooxidation, and electro deionization.
ISS	= International Space Station
ISRU	= In Situ Resource Utilization
FDA	= Food and Drug Administration
GFAAS	= Graphite Furnace Atomic Absorption Spectrometer ThermoFisher
PBS	= Phosphate Buffered Saline
GRAS	= generally recognized as safe
LHS-1	= Lunar Highland Simulant
LDPE	= Low density polyethylene also known as Nalgene
Me	= metal ion
MeO	= metal oxide
PPM	= parts per million
Tween	= polysorbate surfactant

I. Introduction

When NASA returns to the Moon with the Artemis program, the plan is to establish a sustainable infrastructure.[1] In situ resource utilization (ISRU) is defined as the use of local resources to produce space resources and will be crucial for future long-duration missions. Conventional strategies for ISRU often face limitations related to the equipment need for processing, due to their mass and energy requirements, along with the considerable number of consumables necessary. A promising solution to overcome these challenges involves the use of self-reproducing organisms. Unlike traditional methods, the production of these organisms requires minimal resources such as water, a bioreactor, and a readily transportable growth medium.[2] Microorganisms can effectively employ various techniques

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such as biomining for material production and extraction for construction of settlements, infrastructure, and manufacturing.[3] Importantly, microbial production processes do not require complex factories, extensive energy investments, or toxic chemicals.[4] The concept of employing microorganisms for space resource extraction was initially investigated in 2007 during NASA's workshop on lunar regolith biomining.[6] Also, the BioRock experiment was performed on the International Space Station (ISS) to demonstrate how altered states of microgravity and microorganisms influence mineral formation.[5] BioRock was the first test of extraterrestrial biomining and the first use of a miniature prototype of a biomining reactor in space.[5]

Industrial applications of microbes

Currently, the primary industrial application of biomining revolves around the extraction of copper from low-grade ores, constituting a significant portion of global copper production through heap or stockpile bioleaching. Beyond copper, biomining extends its utility to the production of aluminum, cobalt, nickel, zinc, and uranium.[7] Historically, biomining has been utilized predominantly in the processing of sulfide ores and uranium ore.[7] However, advancements have led to the development of laboratory and pilot procedures for silicate and oxide ores, the leaching of processing residues or mine tailings, and the extraction of metals from industrial residues and waste. Filamentous fungi are used in bioleaching due to their ability to secrete organic acids and facilitate the solubilization of metal ions from the solution phase. It has been shown that fungi from Aspergillus and Penicillium genus are among the most effective and important species for biological leaching.[8,9]

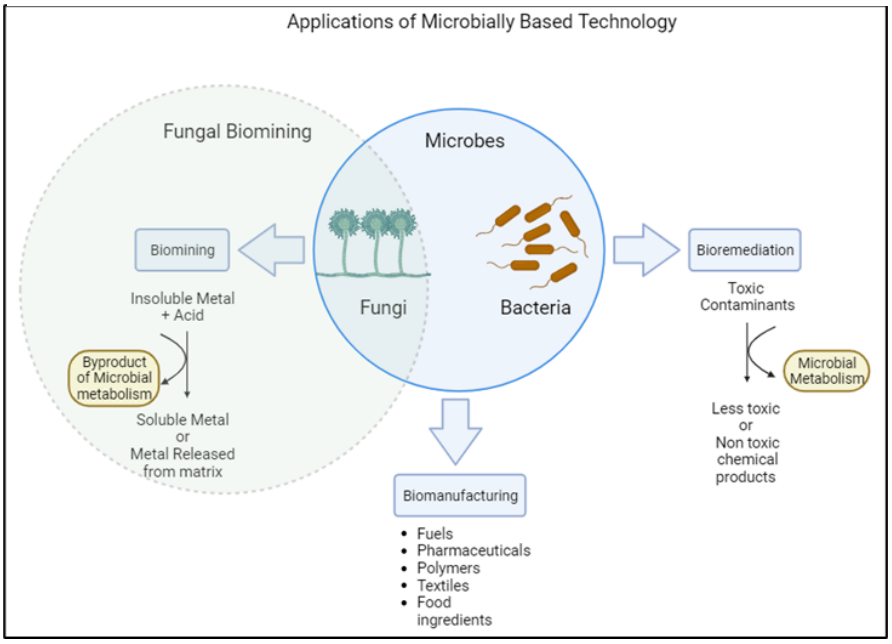

Figure 1. Industrial applications of microbes. Biomining, biomanufacturing, and bioremediation. Each application leverages the metabolic pathways of microbes to transform materials or the microbial byproducts for metal extraction

II. Introduction *Aspergillus Foetidus*:

Aspergillus Foetidus is a filamentous fungus from within the Aspergillus genus. These genera of fungi are spore-formers with diverse metabolic processes that enable them to survive in low-nutrient environments. Filamentous fungi excrete enzymes such as organic acids into the surrounding environment so that substrates can be broken-down into smaller and less complex molecules that can be metabolized by the fungi. These fungi are classified as a biosafety level 1, and the products produced by Aspergillus strains are designated GRAS (Generally Recognized As Safe) by

the Food and Drug Administration (FDA). The ease at which these strains are cultured, their low risk to human health, and their well-documented use in industrial processes make this an ideal microbe to use in biomining processes.

***A foetidus* culture**

Figure 2. *A foetidus* culture on potato dextrose agar.

Aspergillus fungi are a group of acidophilic heterotrophic fungi currently used in metal recovery from industrial wastes such as coal fly ash, E-waste, spent batteries, and spent petroleum catalysts.[10] Metals such as Al, Ni, Zn, Cu, Cd, Cr are extracted by leaching so that they can be used in manufacturing or for bioremediation processes.[10] Microbial-based metal extraction techniques provide new avenues for recovering valuable metals, recycling industrial waste, and remediating the environmental impact of industrial processes.

Aspergillus fungi are ideal microbial candidates for biomining applications in space due to their unique microbial metabolism, tolerance for acidic conditions and high concentrations of heavy metal ions, resistance to radiation, and ability to remain viable in harsh conditions. Acidophilic fungi can tolerate a wide range of pH levels. *A. foetidus* has optimal growth at pH 5.5 and can tolerate a pH range between 2-8.[12] This fungi also has the ability to remain viable in broad temperature ranges from 4 to 80°C with the optimal growth seen between 37°C and 50°C.[13] Experimental work investigating recycling municipal solid waste and fly ash shows that some strains of Aspergillus fungi can withstand aluminum concentrations as high as 3500 mg/L and 3208 mg/L in multi-metal solutions.[10]

Spore forming fungi and bacteria are known to be resistant to radiation and a number of their stress factors such as heat, desiccation, and starvation conditions as part of their survival strategy, which makes these types of microbes attractive for space applications. Spore forming microbes are ubiquitous in terrestrial environments and are now commonly isolated from the interiors and exteriors of spacecraft. Within the same genus, *Aspergillus niger* was found to be one of the primary contaminants in the ISS[25] as a result of numerous microbiological surveys conducted aboard the ISS to determine which microbial species are present and their relative abundance. Contamination control studies have shown that *A. niger* spores can withstand high doses of X-ray (LD90 = 360 Gy) and cosmic radiation (helium-ion LD90 = 500 Gy; and iron-ion LD90 = 100 Gy and it was noted at *A. niger* spores are highly resistant to UV-C radiation (LD90 = 1038 J/m^2), which is significantly higher than that of other radiation-resistant microorganisms. This study suggests that *A. niger* spores may not be quickly inactivated by space radiation.[14] *A. niger's* ability to survive in hostile low nutrient environments such the ISS is attributed to the altered gene expression that is triggered by the stresses caused by enhanced radiation and microgravity conditions. The altered gene expression results in the synthesis of bio reactive compounds that confer survival advantages to the microbes when exposed to environmental stresses. A comparative study performed on *A. niger* strain JSC-093350089 that was isolated from a swab used to sample the US compartment of the ISS and ground-grown *A. niger* JSC-093350089 revealed that the ISS grown strain contained genetic variations, localized mutation distributions, and altered secondary metabolite production.[26] The observed genotypic changes are attributed to exposure to ISS conditions. Additional studies using another spore forming fungi *Aspergillus pullulans*, have shown the ability of fungi to not only to survive exposure to open space, but also determined that these microbes were *already* present on and in the spacecraft. *A pullulans* was isolated from the outer surface of the radiator of the ISS temperature control system and in dust samples collected from the outer surface of the ISS. Using the ISS as an experimental platform rods mounted with test samples were placed outside of the ISS and exposed to outer space conditions with exposure durations of 1 and 2 years, *A. pullans* was shown to increase resistance to radiation after 2 years of exposure to open space near the ISS.[27]

III. Leaching and Precipitation

Fungal biomining comprises four processes: acidolysis, complexolysis, redoxolysis, and bioaccumulation.[15] The fungi's metabolic processes and secondary metabolites mediate these processes. The metabolites produced by the fungi determine the biomining that occurs.[16] Acidolysis is an indirect form biomining mechanism of acidophilic fungi. This process leverages the acidic environment created by the metabolites (organic acids) produced as a byproduct of the fungi's metabolic processes. In this process, protons from the organic acids weaken the bonds in the mineral matrix,

facilitating metals' mobilization (Equation 1). In this way the acidic environment facilitates the leaching of metals from the mineral matrix, which allows the metals to be released into solution so that they can be more easily extracted using other methods.

The remaining charged particles from the organic acid in solution facilitate complexolysis, which involves the interaction between charged particles and the metal ions in solution. This reaction causes the precipitation of metals within salts, facilitating metal separation and extraction. The resulting metal complexes are more stable than the metal ions, which minimizes the toxic effect of metal ions on microorganisms (Equation 2).

Bioaccumulation is another avenue for biomining applications. Instead of releasing metals into solution via degradation of the surrounding matrix, metals are accumulated inside of the microorganisms, which then can be harvested so that the metals can be collected. Bioaccumulation involves the transport of metal ions across cellular membranes via chelation. Chelation is the formation of bonds between organic molecules and metal ions which facilitate electron transfer needed for the microbial metabolic processes.

Redoxolysis oxidation and reduction reactions facilitate fungal leaching via the biochemical interactions between the solid mineral matrixes. The resulting oxidation and reduction reactions result in the release of metal ions in solution (Equation 3).

Due to the overlapping nature of metabolic processes in living cells, the aforementioned metabolic processes co-occur. Multiple factors influence which metals are leached from the provided substrate. Chemical properties of the culture: pH, redox potential, substrate density, the chemical composition of the leached substrate, and the environmental requirements for the microbe being used.

The complete metabolic processes of *A. foetidus* are still being elucidated. However, it is well known that organic acids, citric acid, oxalic acid, and gluconic acid are byproducts of its microbial metabolism. This fungi's unique ability to produce high levels of organic acid production accounts for its importance in the industrial production of citric acid, metabolites, fungal enzymes, and heterologous proteins.[17] In this experiment, fungi are grown in sucrose media, which provides a carbon source to fuel the microbe's metabolic processes. The fungal metabolism facilitates the production of organic acids, creating an acidic environment that can facilitate aluminum solubilization or leaching from acidified lunar simulant. This process eliminates the need for mineral strong acids sulfuric acid, nitric acid, and hydrochloric acids used in traditional mining practices, which reduces the risk to personnel and contamination of the surrounding environment.[18]

$$Me^{2+} + 2H^{+} \rightarrow Me^{2+} + H_2O$$

Eq. (**1**): Example of an acid leaching reaction where MeO represents metal oxide.

$$Me^{2+} + C_6H_8O_7 \rightarrow \quad Me(C_6H_8O_7)^{-} + 3H^{+}$$

Eq. (**2**): Example of chelation/complexation reaction where Me represents metal ions.

$$Fe^{3+} + 0.5HO_2C\ CO_2H \rightarrow Fe^{2+} + H^{+}CO_2$$

Eq. (**3**): Example of a redox reaction where iron is reduced.

IV. Material and Methods

A. Chemicals

Potato Dextrose Agar (70139) was used to prepare the fungal subculturing plates. Sucrose media was prepared using the following reagents purchased from Sigma Aldrich sucrose (84097), $NaNO_3$ (S5022), KH_2PO_4 (P9791), $MgSO_4$ x 7H20 (M2773), KCl (P9541) and yeast extract (92144). 20X PBS with 1% tween was diluted to 1X for spore collection to prepare the fungal inoculum.

LHS-1 Lunar Highland simulant was purchased from Exolith lab. The composition of the lunar simulant by percent weight is as follows: SiO_2 (51.2), TiO_2 (0.6), Al_2O_3 (26.6), FeO (2.7), MnO (0.1), MgO (1.6), CaO (12.8), Na_2O (2.9), K_2O (0.5), P_2O_5 (0.1), and LOI (0.4). Relative abundances were measured using x-ray fluorescence (Exolith Website).

Biomining process map

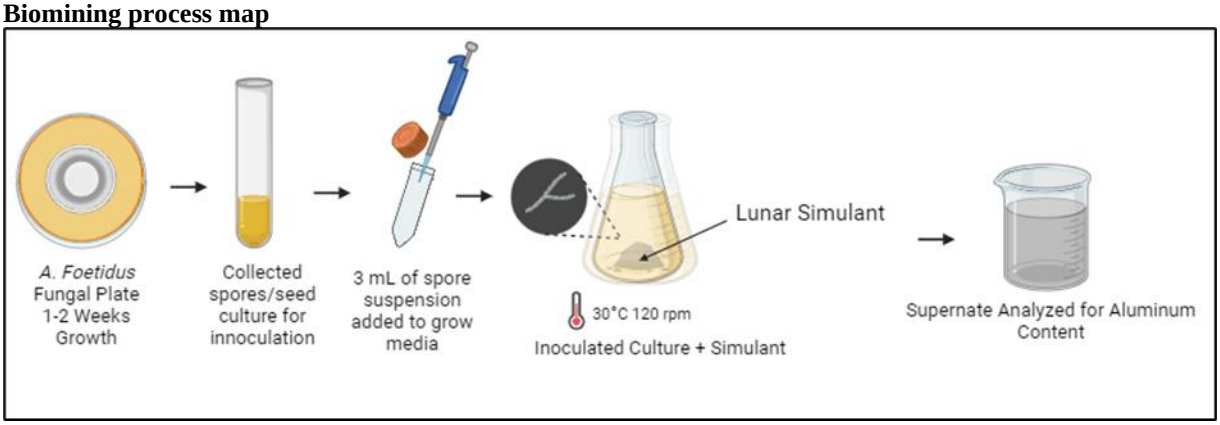

Figure 3. Biomining process map. *A. foetidus* cultured on potato dextrose agar plates is used to create a spore suspension that is then used to inoculate sucrose media. Once the inoculated fungal culture reaches ideal pH lunar simulant is added so that the biomining process can be begin. Supernatant is collected and analyzed for aluminum content.

B. Fungal strain and growth conditions and preparation of lunar simulant

A. foetidus (ATCC 16878) was obtained from American Type Culture Collection as a freeze-dried culture. *A. foetidus* laboratory stock was maintained using subcultures on potato dextrose agar plates and kept in an incubator at 30˚ C.

25 grams of LHS-1 Lunar Highlands Simulant were weighed using an OHAUS PX84E analytical balance and wrapped in two layers of UHV ultrahigh-grade aluminum foil. The wrapped simulant was then autoclaved[8] for 1 hour at 110˚C.

C. Spore Inoculum Preparation

Laboratory stock cultures of *A. foetidus* were submerged in 1 x PBS with 0.1% Tween to recover the spores for the preparation of the spore inoculum. Each grow plate was flooded with 10 ml of 1 x Phosphate Buffer Solution (PBS). The plates were then scraped with an autoclaved cell scraper to release spores into the buffer solution. The buffer with suspended spores was then filtered using a sterile Corning Cell Strainer (REF431750) 40 µm cell strainer into an autoclaved glass beaker. The concentration of the spore suspension was determined using a hemocytometer[9] and a microscope[10]. The spore inoculum was adjusted to obtain a minimum concertation of 1 x 10^7 spores per milliliter. The standardized spore suspensions were stored in sterile 50 ml falcon tubes and refrigerated at -20˚C. For biomining experiments 1 ml of spore suspension was added per 100 ml of sucrose medium. Sucrose medium (g/L): sucrose: 80 g, $NaNO_3$: 1.5 g, KH_2PO_4: 0.5 g, $MgSO_4$.7H20: 0.025 g, KCl: 0.025 g, and yeast extract: 1.6 g. Media was filter sterilized using a Corning .22 µm filter.

[8] Market Forge Sterilmatic Autoclave
[9] Quick Read Precision Grid Urinalysis Slide with Cell Counting grid CAT3800
[10] Amscope 40X- 1000X Upright Fluorescent Microscope

D. Two-Step Biomining Experiment

A foetidus biomining culture

<u>Figure 2a and 4b.</u> Pictured on the left 4a *A. Foetidus* fungal culture after addition of lunar simulant (day 1). Pictured on the right 4b *A. foetidus* fungal culture with lunar simulant three days after addition of lunar simulant.

Two-step bioleaching is performed using 1L autoclaved Erlenmeyer flasks. Each biomining culture comprises 5 ml of fungal inoculum (with a spore concentration of 2.32 x 10^7 spores per ml) added aseptically to 500 ml of sucrose media. The experimental control was sucrose media with lunar simulant with no spore inoculum added—the inoculated flasks were then placed in an Innova Incubator Shaker 4430 at 30˚ C at 120 rpm. The flasks are monitored for pH to identify when the ideal pH of 5.5 was reached approximately two days after inoculation. Twenty-five grams of autoclaved Lunar Highland simulant was added to all the flasks once the inoculated cultures reached an ideal pH of 5.5 or lower. An OAKTON pH 550 meter was used to monitor the inoculated biomining cultures. All flasks were returned to the incubator shaker at 30˚ C at 120 rpm for 14 days using sterile pipettes. The inoculated cultures were then sampled on days 1, 4, 8, 12, and 14. Inoculated flasks were allowed to settle for several minutes before each sampling event to minimize the simulant in the collected sample. For each sampling event, 10 ml of culture was aseptically collected and stored in sterile 14 ml FALCON tubes and placed in the freezer at -20˚C until analysis could be performed. At the end of the experiment, all experimental flasks were removed from the shaker incubator. The cultures were then allowed to settle overnight to achieve a clear separation between the spent simulant and fungal biomass. The remaining supernatant was then collected aseptically using sterile pipettes, which were then filter sterilized using .22 µm filters. The filtered supernatant was then stored in sterile autoclaved glass bottles until analysis could be performed.

E. Test 1: Determination of Aluminum during *A. foetidus* growth

During the bioprocess, the flasks contained an heterogenous mixture of *A. foetidus* biomass LHS-1 simulant and liquid solution that was continually stirred. Periodically the flasks were moved temporarily in a fume hood and particles were left to settle for a few minutes. During the *A. foetidus* growth, the flasks were sampled 5 times by pipetting using sterile Eppendorf[11] 10 ml serological pipettes and 10 mL of the culture were transferred to 15 mL conical sterile polypropylene centrifuge tubes and stored at 4 ˚C. These samples were diluted by weight using a OHAUS PX84E analytical balance 30-120 times using ultrapure ELGA water in 125 mL using Low Density Polyethylene (LDPE) narrow mouth bottles and processed using ultrapure Optima brand nitric acid[12] (HNO_3) 5% v/v and 1% hydrogen peroxide (H_2O_2)[13] to allow sample digestion. Al concentration in H_2O_2 was checked and it was found negligible 1 ppb in a 1% H_2O_2 deionized water solution. Theses samples were stored at ambient temperature for three days before analyses. Al determinations were performed using a Graphite Furnace Atomic Absorption Spectrometer (GFAAS) using a secondary wavelength of 394.4 nm to reduce sensitivity. Al quantification was performed by bracketing the relatively high Al concentrations using three single standards solutions and one blank. Procedural blanks were found to be negligible, less than 1 ppb, with precision estimated at the 2% level. However, accuracy was within 38%, which falls at the lower end of the range of Al concentrations in the samples. The prescribed value of Al[14] was 33.9 ppb, while the obtained Al value was 53 ppb. Results are illustrated in Table 1 the standard deviation reflects the dispersion of two replicates per sample.

[11] Eppendorf catalog 0031027722
[12] Optima Fisher Scientific 1% v/v volume of acid per 100 units of the total volume acid with water.
[13] Fisher Chemical, 50% v/v volume of hydrogen peroxide per 100 units of the total volume hydrogen peroxide with water.
[14] Certified Material SLRS-6, National Research Council of Canada

Aluminum concentration determined during *A foetidus* growth

Faetitus Sample	Day	Al (ppm)	STD (ppm)
6/23/2023	1	633	11
6/27/2023	4	996	10
7/1/2023	8	4604	32
7/5/2023	12	4265	26
7/7/2023	14	6174	49

Table 1. Aluminum concentration determined in samples taken from the flask during the growth of the *A. foetidus*.

F. Test 2: Determination of Aluminum in the different phases of the bioreactor

At the end of the process of growth of *A. foetidus*, the flasks 1 control flask and 3 replicate biomining cultures were left to settle and then were sampled for the liquid and the solid biomass phases deposited on the bottom of the flasks. Approximately 150 mL of supernatant was extracted from each flask and filtered using sterile Corning Cell Strainer[15]. A 1 mL volume of the filtrate was transferred to 10 mL vials, diluted 125 times by volume in 125 mL LDPE bottles and acidified using ultrapure HNO_3. Two fractions of the *A. foetidus* biomass shallow and deeper were extracted by using a serological pipette to pull off supernatant. Approximately 1 g of each of the two fractions of *A. foetidus* were sampled and diluted 125 times by weight in 125 mL LDPE bottles and acidified using ultrapure HNO_3 (Optima Fisher Scientific 1% v/v) The remaining liquid fraction approximately 140 mL was poured back in the flasks and added to the regolith that was left on the bottom after the process. 1 mL aliquot of these solutions were diluted 125 times by volume in 125 mL LDPE bottles and acidified 1 % using ultrapure HNO_3. All processed samples were stored at ambient temperature for three days before chemical analysis. The determination of Al concentrations by GFAAS were performed using the same method described in Test 1.

Aluminum concentration determined at the end of *A foetidus* growth

Sample	Al (ppm)	STD (ppm)
Control solution (no growth)	3.3	0.2
Filtered solution	37	3
Unfiltered solution	656	265
Faetidus mass	10151	2039
Faetidus + Regolith	13634	1879

Table 2. Aluminum (Al) concentration determination of the phases sampled from the flasks at the end of the *A. foetidus* growth.

V. Conclusion

The experiments indicate that Aluminum concentration in the solutions increased over time. As a result, the use of biomining and aluminum may have diverse applications on the moon, ranging from the creation of power transmission lines to a solar power-collecting grid to the construction of spacecraft components. The moon's abundant aluminum resources stem from its presence in anorthite, a mineral that constitutes a significant portion of the lunar crust.[28]Anorthite, the aluminum-rich end member of the plagioclase series, is a key contributor to lunar aluminum. In its pure form, with the chemical formula $CaAl_2$ $Si2O_8$, it contains 19.4 wt. % aluminum metal, 20.2 wt. % silicon metal, 14.4 wt. % calcium metal, and 46.0 wt. % oxygen. Compared to Earth's crust, the lunar crust boasts a notably higher proportion of anorthite, with the lunar highlands being particularly abundant in this mineral.[21,22,23]Noteworthy is the variability in normative anorthite content across lunar highlands, with the Apollo 16 site standing out as the region richest in anorthite. Lunar soil, primarily composed of fine particles resulting from meteorite impact comminution of lunar rocks, presents a strategic advantage. Its pre-pulverized state, with a mean grain size ranging from 40 to 100 µm, facilitates easy extraction through simple surface mining techniques. This contrasts with the coarser rock or megaregolith beneath the fine-grained regolith, which would demand more complex mining processes and energy-intensive grinding equipment. From an economic standpoint, lunar soil emerges as the optimal source of anorthositic aluminum ore. Its readiness for extraction and processing implies reduced operational complexities

[15] Corning Cell Strainer REF431750.

compared to the underlying megaregolith. To illustrate the feasibility of utilizing microorganisms for ISRU, our focus has been on Lunar Highland simulant as the initial substrate.

The benefits of microbially based technologies being developed on Earth will be vital in supporting the long-term human presence in space, where increased efficiency, lower energy requirements, and lower risk to personnel will be crucial in developing the extraterrestrial infrastructure needed to provide resources for astronauts. Biologically based systems offer a viable option for ISRU in space. This is particularly crucial in environments where resupply and initial material constraints are significant factors. Microbially based technologies have the added benefit of being regenerative, self-replicating, and multipurpose. These are crucial characteristics for any technology used in the closed-loop life support systems are needed in space. While this paper will focus on the biological extraction of metals using fungi, microbially based technologies can also be leveraged to produce pharmaceuticals, fuel, oxygen, polymers, and waste recycling.[24]

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