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Biological leaching of copper, zinc, and cobalt from pyrometallurgical copper slags using *Aspergillus niger* and *Penicillium ochrochloron*

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ABSTRACT

As a flexible and environmentally friendly technology, bioleaching is widely used to recover base metals from raw materials and industrial waste. This study aimed to test and compare the bioleaching potential of the fungi *Aspergillus niger* and *Penicillium ochrochloron* for copper, zinc, and cobalt in a weathered copper slag sample collected from Eliseyna, Bulgaria. Three techniques were employed: direct (one- and two-step) bioleaching of copper slag in the presence of the relevant fungal species, and indirect leaching using spent medium obtained after a week of preliminary fungal cultivation and microbial biomass separation. The results showed that a 5% pulp density was optimal for all bioleaching techniques, with 28 days being the optimal duration for direct techniques and 3 days for indirect techniques. Zinc and cobalt bioleaching efficiencies using *A. niger* by direct techniques were higher than those using *P. ochrochloron*, reaching 65.3% and 76.8%, respectively. However, the copper bioleaching efficiency was only 8.3% due to selective precipitation. Significant co-leaching of iron and silicon affected the surface and mineralogy of the copper slag particles. In conclusion, *A. niger* exhibits higher bioleaching potential for zinc and cobalt in copper slag than *P. ochrochloron*, with direct and indirect mechanisms playing nearly equal roles in their leaching.

Keywords: copper slag, fungi, non-ferrous metals, bioleaching.

INTRODUCTION

Bioleaching is an environmentally friendly and sustainable method for unlocking and recovering non-ferrous, rare, and precious metals from raw materials with diverse mineralogy and chemical properties. It relies on bacteria, archaea and fungi growing in various environmental factors, such as temperature and pH. Bioleaching mechanisms involve direct contact between microbial cells and solid surfaces, or indirect contact via microbial metabolites with oxidative, reductive, acidic or alkaline properties [1, 2]. Therefore, bioleaching is a flexible technique that can be adapted to the specific requirements of the solid sample and the raw materials it contains. Operating at ambient pressure and temperature enables the technique to achieve low investment and operational costs, which are the main advantages compared to conventional technological alternatives for processing raw materials [3–5]. Initially, bioleaching was used for processing low-grade ores, ore pretreatment, and impurity removal [6–8]. Its effectiveness has been proven on an industrial scale, making it an economically attractive and environmentally friendly way to process copper, uranium or gold-containing ores.

The electronics and information technology industries, as well as the green transition, have significantly increased the global demand for metals and other raw materials [5, 9, 10]. Furthermore, the limited global stock in the Earth’s crust and the uneven distribution of some of these elements led to their inclusion on the critical raw materials list [11]. Consequently, many bioleaching studies currently focus on processing various types of solid materials, such as mine tailings [12], solid waste [13–15], fly ash [16], electronic scrap [17, 18], and spent catalysts [19]. It is due to the significant amounts of waste deposited historically or generated annually by current industrial activity, as well as their content of base metals, rare earth elements, or noble metals. For instance, 1.5–2 tons of solid waste, slag or red mud are produced for every ton of copper concentrate or bauxite ore processed, respectively, and these have specific mineralogy, chemical content and properties [20, 21].

Copper slag is a by-product of the copper smelting industry. The content of non-ferrous metals (such as copper, zinc and cobalt) depends on the origin of the ore and the industrial processes involved in its formation [20, 22]. Currently, there is no sustainable method for extracting and recovering these metals from copper slag, and it is common practice to dispose of it in landfills. Some authors [23–26] have studied the chemical leaching of slags using acids (sulfuric and nitric acid), bases (ammonia) and chemical oxidants (Fe^{3+}). Slag leaching at highly acidic pH is a promising process for recovering raw materials. However, the substantial operational costs associated with this approach are proving to be a significant obstacle to scaling up results on an industrial level. Studies on the bioleaching of copper slag using acidophilic chemolithotrophic bacteria and archaea belonging to the genera *Acidithiobacillus*, *Leptospirillum*, and *Sulfobacillus* revealed significant leaching of non-ferrous metals when elemental sulphur was added to the nutrient medium. The ability to produce sulfuric acid, which enhances the extraction of base metals through the acidolysis of minerals, and the direct or indirect bacterial oxidation of sulfides [25, 26] are the main advantages of bacterial leaching at acidic pH. However, the subsequent processing of generated leach solutions is complicated by the high concentrations of co-leached iron and silicon [27]. Another option for processing slag by bioleaching is to use heterotrophic bacteria and fungi in aerobic conditions. Several studies [8, 28, 29] have demonstrated that fungi (e.g. *Aspergillus*, *Penicillium* and *Trametes*) have a greater leaching potential than bacteria (e.g. *Pseudomonas*, *Bacillus* and *Sporosarcina*) due to their ability to produce organic acids such as citric, oxalic, gluconic and malic acids, which facilitate base metal leaching and sequestration through acidolysis and the formation of complexes with different solubilities [30, 31].

This study aims to test and compare the bioleaching potential of the fungi *Aspergillus niger* and *Penicillium ochrochloron* for copper, zinc, and cobalt in copper slag from Eliseyna, Bulgaria.

MATERIALS AND METHODS

Copper slag characteristics

The copper slag sample was collected from the surface of the weathered zone of a dump near Eliseyna, Northwestern Bulgaria. The sample was transported to the laboratory in a dust-free bucket and stored at a lower temperature for subsequent use. The original copper slag sample had rounded particles between 150 µm and 10 mm, which were ground to a dominant particle size of 75 µm to 25 µm. In that form, the copper slag was used in (bio)leaching experiments. A representative sample of copper slag was subjected to suitable digestion, after which the content of chemical elements in the liquid obtained after digestion was analysed by atomic absorption spectrometry and inductively coupled plasma. The mineralogical composition of the copper slag was determined by the X-ray Diffraction analyser EMPYREAN, which utilised Cu-Kα radiation and operated at 40 kV and 30 mA. The surface morphology of the copper slag was determined using an electron microscope (JEOL 6390) with an INCA Oxford EDS detector. The copper slag pH was determined in distilled water after one hour of mixing with an overhead mixer at 150 rpm.

Chemical leaching with sulfuric acid

The consumption of acid and the chemical leaching of non-ferrous metals were studied through the serial addition of sulfuric acid to the prepared pulp, under agitation at 300 rpm and a temperature of 25 °C. The addition of acid was terminated once the pulp's pH level reached the predetermined target values (5.0, 4.0, and 3.0), and the value was constant for a period of six hours. The pulp sample was collected and subjected to centrifugation, after which the analytical methods described above were employed to determine the concentration of elements in the transparent solution.

Fungal strains and bioleaching techniques

Aspergillus niger was obtained initially from the Microbial Culture Collection at the Department of General and Industrial Microbiology at the University of Sofia. *Penicillium ochrochloron* CCM F-158 (hereinafter referred to as *P. ochrochloron*) was obtained from the Czech Collection of Microorganisms at the Department of Experimental Biology at Masaryk University. The strains were cultivated on Czapek Dox medium by sub-culturing at 25 °C and were preserved at 4 °C when required. The nutrient medium employed in the bioleaching experiments comprised sucrose (185 g/L), KH_2PO_4 (3.0 g/L), NH_4Cl (0.96 g/L), $MgSO_4.7H_2O$ (1.2 g/L), and trace amounts of $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, and $CuSO_4.7H_2O$. The medium was inoculated with 10^6 spores/ml and then incubated for seven days at 25 °C with continuous shaking at 150 rpm. Except for the duration, all other elements were consistent across all experiments. The present study investigates the biological leaching of non-ferrous metals from copper slag, utilising both direct and indirect techniques within a batch mode of operation. The copper slag sample used in these experiments underwent preliminary sterilisation at 2 atmospheres pressure and 180 °C for 2 hours. Control experiments were conducted utilising fresh nutrient medium and sterile copper slag. All experiments were run in three repetitions, and the results were an average of the mean value ± standard deviation.

The direct one-step leaching technique is employed. Each variant was inoculated with a homogenised spore suspension containing 10^6 fungal spores per milliliter. Evaporation-caused water loss was compensated for by adding distilled water. Pulp pH and the concentrations of leached elements in the filtrate were measured weekly. When the experiment ended, copper residue and biomass were washed with distilled water until the filtrate was transparent. The biomass and copper slag residue were dried at 80 °C for 24 h, cooled in a desiccator and weighed. The biomass was removed from the copper slag residue by ashing at 500 °C, and the difference between the weighted sample before and after the ashing was the exact biomass value. Direct two-step leaching technique: The first step was cultivating the fungal species in Czapek-Dox medium for a week to build up biomass. The addition of copper slag, related to the relevant pulp density, resulted in a transition from the first to the second step of bioleaching. The monitoring applied during the bioleaching test and the residue processing protocol were identical to those described for direct one-step bioleaching. After a week of cultivation, the value of fungal biomass was determined in some variants by centrifugation aseptically at 10.000 rpm for 10 minutes.

The indirect leaching technique was performed using the spent medium obtained after cultivating the fungal species on Czapek-Dox medium for one week. Titration and spectrophotometric methods determined the citric and oxalic acid concentration in the spent medium [32, 33]. The Metrohm 718 STAT Titrino titrator used 0.2 N NaOH and a pH end of 8.5 to determine acidity. The study examined the indirect bioleaching of non-ferrous metals from copper slag at 5, 10 and 15% pulp densities. The experiments lasted five days.

Mass balance

The recovery of non-ferrous metals, iron, and silicon from copper slag through fungal bioleaching under the relevant experimental conditions was calculated, taking into account the difference between each element's initial and residual content, as well as the calculated weight reduction of the copper slag sample.

RESULTS AND DISCUSSION

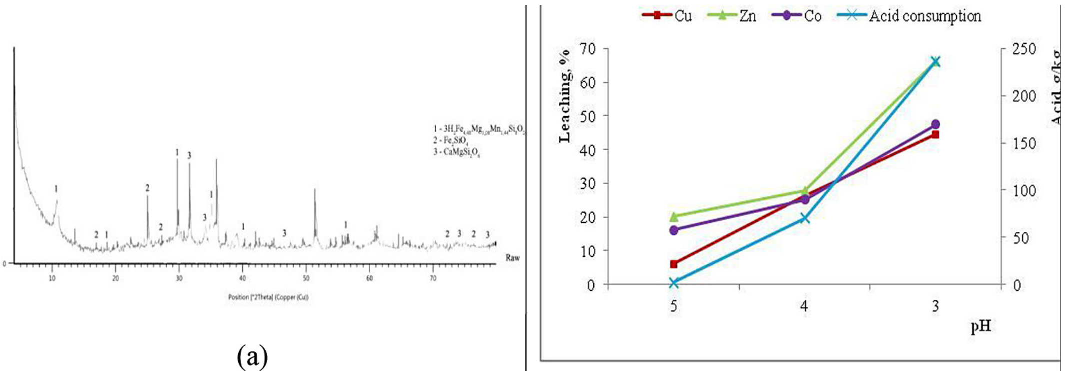
The copper slag sample collected from a dump at Eliseyna, a village in Northwestern Bulgaria, was studied. The results of the physicochemical and chemical analyses (Table 1) indicate that the sample contains copper, zinc, and cobalt in concentrations of 0.36%, 1.93%, and 0.09%, respectively, along with higher amounts of iron and silicon. This sample is used in studies for non-ferrous metals bioleaching recovery.

An X-ray diffraction study of the copper slag sample (Figure 1a) reflected a mineralogical pattern of slags with fayalite (Fe_2SiO_4) and the presence of an unidentified type of clinopyroxene ($Ca_{0.949}Fe_1Na_{0.051}O_6Si_2$).

The studied sample from Eliseyna contain protomangano-ferro-anthophyllite ($H_2Fe_{4.48}Mg_{1.08}Mn_{1.44}O_{24}Si_8$) with a 30% share, not the goethite and magnetite described as typical for copper slag. This finding suggests that a weathering process occurred during the storage

Table 1. Characteristics of the copper slag sample used in this study

Sample	pH (H_2O)	Content, %									
		Cu	Zn	Co	Fe	Si	Ca	Mn	Al	P	S
F	8.81	0.36	1.93	0.09	27.2	15.9	6.9	0.8	2.78	0.08	0.91



(a)

(b)

Figure 1. XRD pattern of copper slag (a); sulfuric acid consumption and non-ferrous metals in dependence on the target pH (b)

period, which spanned over six decades. Weathering involves altering the copper slag mass into smaller grains with a higher surface area. Chemical changes also occur due to leaching, co-precipitation and adsorption. This process leads to the formation of secondary mineral phases, like oxides, oxyhydroxides, and carbonates, in copper slag dumps (10).

Acid consumption and the non-ferrous metals leaching

The chemical composition and the surface area of the finely ground copper slag sample determine its alkaline properties and significant acid consumption (Figure 1b), especially if the target pulp's pH is lower than 4.00. The chemical composition and mineralogy of the copper slags are similar to those formed during steel and other non-ferrous metal production. Therefore, sulfuric acid consumption is roughly the same, at around 250 kg per ton of slag when the target pH is 3.0. [31, 34, 35]. The mineral acidolysis and dissolution process is pivotal in determining the non-ferrous leaching of the copper slag sample. It has been observed that the respective values exhibit a direct correlation with an increase in the amount of sulfuric acid present. The leaching of secondary minerals, including iron oxides and carbonates, is critical in determining the value of acid consumption at a slightly acidic pH. Conversely, the leaching of fayalite and diopside governs acid consumption at highly acidic pH levels. As indicated by other researchers' findings, most non-ferrous metals (zinc, cobalt, nickel) have been observed to be disseminated uniformly within copper slag silicates [24, 36]. The results demonstrate that 20.2%, 16.1% and 6.1% of zinc, cobalt and copper are leached from the copper slag sample at a pH of 5.0.

Furthermore, the process efficiency increases to 66.3%, 47.5% and 44.6%, respectively, when the pH is reduced to 3.0. It is evident that at a pH of 1.5, 75.8% and 86.7% of copper and cobalt are leached. Furthermore, it is notable that zinc leaching (77.2%) is most significant at pH 3.0. However, non-ferrous metal recovery is concomitant with substantial iron and silicon co-leaching. For instance, 68.9% and 48.5% of iron and silicon, respectively, are leached at a pH of 3.0. Silicic acid (H_4SiO_4) is a silicon form at an acidic pH that readily transforms into silica gel (SiO_2). The higher surface area and surface charge of the latter determine some losses of non-ferrous metals due to their adsorption. The leaching test reveals that approximately a quarter of copper is not leached with sulfuric acid, indirectly demonstrating that the base metals exist as refractory to acidic leaching forms, sulfides and zero-valence copper, in the studied copper slag sample.

Mechanism of base metals leaching from copper slags using fungi and/or their metabolites

Fungi have been identified as a significant bio-weathering agent, a property attributable to the secretion of organic acids in the environment [37]. These organisms are employed extensively in the bioprocessing of various raw materials [38]. The study will demonstrate how various bioleaching techniques (with or without the presence of *A. niger*/*P. ochrochloron*) impact the recovery of base metals and the co-leaching of iron and silicon from copper slag at a slightly acidic pH.

It is evident from the chemical content, XRD, and acid consumption results that the base metals in the copper slag sample are susceptible to bioleaching due to the growth and activity of the fungal species by the following set of reactions:

1. Organic acids production and subsequent secretion in the milieu:

$$C_{12}H_{22}O_{11} + 3.5O_2 \rightarrow 2C_6H_8O_7 + 3H_2O \quad (1)$$

$$C_{12}H_{22}O_{11} + 3O_2 \rightarrow 6(COOH)_2 + 5H_2O \quad (2)$$

2. Deprotonation of organic acids:

$$(COOH)2 \rightarrow (C2O4)2- + 2H+ \quad (3)$$

3. Proton attack and mineral acidolysis:

$$Si\text{-}O\text{-}M + H+ \rightarrow Si\text{-}O\text{-}H + M+ \quad (4)$$

$$Fe_2SiO_4 + 4H^+ \rightarrow 2Fe^{2+} + H_4SiO_4 \quad (5)$$

4. Complexolysis: the formation of complexes between cations and organic anions, which possess different solubilities:

$$Zn^{2+} + 3C_6H_7 0_7^- \rightarrow Zn(C_6H_7O_7)_3^- \quad (6)$$

$$Cu^{2+} + C_2O_4^{2-} + nH_2O \rightarrow CuC_2O_4.nH_2O \downarrow \quad (7)$$

Organic acid production of *Aspergillus niger* and *Penicillium ochrochloron*

The strain of *A. niger* was selected for the study due to its demonstrated bioleaching potential, which has been extensively evaluated and validated for a broad spectrum of raw materials, including laterite, copper slag, and black shale [8, 39, 40]. The rationale behind incorporating the *P. ochrochloron* strain into the study was derived from its capacity to thrive in an environment with elevated concentrations of copper ions. The synthesis and accumulation of citric acid by fungi are found to be strongly influenced by the composition of the nutrient medium and the conditions (e.g., the rate of aeration and pH) applied during cultivation. The yield of citric acid is optimised when fungi utilise sucrose or monosaccharides as a carbon source in their growth, with a minimal concentration of 50 g/L. Fungi can also be cultivated on certain inexpensive raw materials, such as hydrolysate starch, sugar cane broth, and by-products, including sugar cane and beet molasses, yielding a lower citric acid yield [41].

A. niger and *P. ochrochloron* were cultivated on a nutrient medium optimal for citric acid synthesis, and both species exhibited a similar reaction, with branched microbial biomass formation, displaying a bulbous appearance and distinguishing morphology for each species. Following a week of cultivation under shaking conditions and at a temperature of 25 °C, which were designed to enhance citric acid production, the chemical analyses demonstrated that *A. niger* exhibited a higher rate of sucrose oxidation to citric acid than *P. ochrochloron*. It resulted in the synthesis of a higher amount of fungal biomass and the generation of a spent medium characterised by highly acidic pH due to the higher concentrations of citric and oxalic acids (see Table 2).

Direct one-step technique for bioleaching of non-ferrous metals from copper slag

The copper slag bioleaching with *Aspergillus niger* and *Penicillium ochrochloron* was performed to compare the pattern of non-ferrous metals recovery and to determine the optimal conditions for their leaching. All experiments were conducted in batch operation by inoculating the nutrient broth containing ground copper slag at pulp densities of 5, 10, and 15%, with 10^6 spores/ml of the relevant fungal species. Each experiment was conducted in triplicate. The flasks were incubated at 25 °C under continuous shaking for 42 days, and the pH and leached amount of Zn, Co, and Cu were measured weekly.

Effect of test duration

Both fungal species form biomass with the appearance and morphology indicative of preferential citric acid synthesis 5–6 days after the start of copper slag bioleaching, coinciding with a pH decrease from 8.81 to 4.40 and 4.77 in the experiments with *A. niger* and *P. ochrochloron*, respectively. The biological acidity generation process increases the concentration of hydrogen ions following reaction

Table 2. Spent medium of the studied fungal strains after a week of cultivation

Index	*Aspergillus niger*	*Penicillium ochrochloron*
pH	2.58	3.24
Citric acid, g/ L	24.8	18.9
Oxalic acid, g/L	8.1	10.9
Acidity, g H^+/L	0.94	0.65

2 due to the deprotonation of the carboxylic group, which accelerates the leaching of copper slag minerals through reactions 4 and 5. The pregnant solution and fungal biomass display different shades of green depending on the concentration of ferrous iron. For example, the colour is dark grassy green in the experiments with *A. niger*.

As demonstrated in Figure 2, the bioleaching of zinc and cobalt from the slag sample exhibited a direct correlation with the decrease in solution pH during the initial three weeks of the experiment. The maximum recoveries of zinc and cobalt are attained at the end of the fourth week of cultivation, as the bioleaching potential of *A. niger* is higher compared to that of *P. ochrochloron.* For instance, the pH level attained 3.28 by the conclusion of the fourth week of the experiment, with *A. niger* at a pulp density of 5%, resulting in the leaching of 65.3% of zinc and 76.8% of cobalt. In comparison, the variant with *P. ochrochloron* has a pH value of 4.10, leaching 28.5% of zinc and 37.7% of cobalt. Following the fourth week, the process of acid generation with both fungal species ceases, thus maintaining a constant pH. Concurrently, a decline in non-ferrous metals leaching is observed, a consequence of sorption processes.

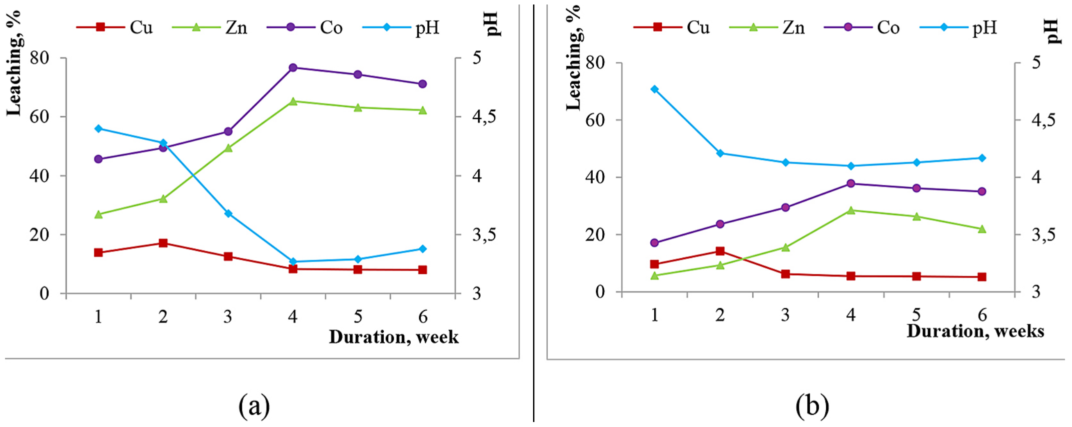
Copper bioleaching follows a different pattern from that of zinc and cobalt. During the experiments, the copper concentration in the pregnant leach solution of both fungal species never exceeded the threshold of 17–35 mg/L, which refers to 0.26–0.55 mM copper. The observed concentration range is significantly lower than the minimum inhibitory concentration value of copper (5.0 mM), as established by other researchers [42]. Several studies have shown that *A. niger* employs various strategies in response to environmental stressors. These strategies encompass biosorption on the surface of fungal biomass, intracellular bioaccumulation, and extracellular metal precipitation, and they are microbial adaptations in response to the leaching of certain elements from the solid sample. For instance, *A. niger* has been shown to accumulate approximately 77% of the lithium leached during battery processing [43], and to undergo physicochemical processes of sorption on its surface [44].

Oxalic acid overproduction has been identified as a protective mechanism employed by fungi in response to elevated bioavailable concentrations of manganese, aluminium, and copper within their microenvironment. In response to these elevated concentrations, fungi adapt by transitioning their metabolism from citric acid synthesis to oxalic or gluconic acid synthesis [45, 46]. The concentrations of metals mentioned above reach 120 mg/L, 727 mg/L and 28.2 mg/L after ten days in the bioleaching experiment with *A. niger*. During this time, the pH drops, coinciding with higher amounts of oxalic acid. For instance, the oxalic concentration at the end of the third week of bioleaching reaches 16.7 and 11.6 g/L, respectively, in experiments with *A. niger* and *P. ochrochloron*. Oxalic acid is a strong diprotonic organic acid with pKa values ranging from 1.25 to 4.14. At a slightly acidic pH, it donates two protons at a higher rate, producing the oxalate anion. This anion forms complexes with alkaline earth metals, non-ferrous metals and rare-earth elements. Their solubility values differed significantly, so the oxalate ions precipitated them sequentially to insoluble oxalates, as shown by reaction 7. It resulted in 8.3% copper leaching when *A. niger* was used and 5.5% in the presence of *P. ochrochloron*. These figures are insignificant compared to the result of base metal leaching with sulfuric acid. The phenomenon is explained by the selective precipitation of copper ions as copper oxalate by fungi due to alterations in the ratio of secreted organic acids. The lower Ksp value of copper oxalate enables it to sequester copper ions from other leached non-ferrous metals and iron. That reaction is widely used to process technogenic solutions [38, 48, 49].

Effect of pulp density

The experiments conducted at 10 and 15% pulp density validate the findings regarding the enhanced leaching of non-ferrous metals from copper slag in the presence of *A. niger* as compared to *P. ochrochloron* (Figure 3).

For instance, the bioleaching of zinc and cobalt with *A. niger* after 28 days at 10% pulp density yielded 32.6% and 38.8%, respectively, while for *P. ochrochloron*, the values were 19.6% and



(a)

(b)

Figure 2. Non-ferrous metals recovery from copper slag by direct one-step bioleaching with *A. niger* (a); *P. ochrochloron* (b) at a 5% pulp density

20.4%, respectively. The values mentioned above are lower than those calculated at 5% pulp density for the relevant fungi species. The values for copper bioleaching with *A. niger* and *P. ochrochloron* are 22.2% and 12.7%, respectively, corresponding to 0.63 mM and 0.36 mM of copper, respectively. Neither value exceeds the minimum inhibitory concentration of copper for fungi. The concentration of manganese and aluminium in the pregnant leach solution increases also, and in the experiment with *A. niger*, the values are 162.4 mg/L and 936 mg/L, respectively. This finding suggests that the elevated levels of manganese and aluminium ions present in the pregnant leach solution do not stimulate further oxalic acid production in fungi beyond the levels observed in experiments conducted at 5% pulp density. Consequently, the maximum level of acid generation through organic acid synthesis and secretion was observed at 5% pulp density for each fungal species. However, in the case of experiments at 10% pulp density of copper slag, the mineral content susceptible to leaching by acidolysis is double that at 5% pulp density. A further increase in sucrose is not possible to boost non-ferrous metal leaching via fungal citric acid production, as it has a negative impact on microbial activity, as shown in [50, 51]. It is thus evident that the limited amounts of secreted organic acids determine the lower rate of mineral acidolysis (as described in reactions 4 and 5) due to the scarcity of protons and the higher value of the final pH. For instance, the pH level at the conclusion of the experiment with *A. niger* is 4.43. The weight of fungal biomass measured at 10% pulp density (9.5–10 g dry weight/L of *A. niger* and 8.5–9.0 g/L of *P. ochrochloron*) is comparable to that measured with the same species at 5% pulp density.

Furthermore, the biomass for each species exhibits its characteristic morphology. The findings indicate that the increased mineral particle content during the experiment and the pregnant solution content do not directly impact fungal growth, as evidenced by the doubling of pulp density. However, it has been demonstrated to affect fungal metabolism and the products released into the solution.

The bioleaching of non-ferrous metals with tested fungal species exhibits a further decline at 15% pulp density. Consequently, the outcomes of zinc, cobalt, and copper bioleaching with *A. niger* cultivation are 23.8%, 31.1%, and 13.8%, respectively. A similar trend of worsened bioleaching of raw materials with fungi from different solid samples has also been determined by other researchers [5, 17, 43]. One of the main disadvantages of bioleaching with heterotrophs compared to that with acidophilic chemolithotrophic bacteria and archaea is that the latter carry out bioleaching of non-ferrous metals at higher rates, even at a 15% pulp density.

Direct two-step technique for bioleaching of non-ferrous metals from copper slag

The primary advantage of the direct two-step bioleaching technique over the one-step technique is that it commences with a substantial amount of fungal biomass. This accelerates the oxidation of the carbon source in the nutrient medium to citric acid and other organic acids, thereby accelerating the rate of non-ferrous metals bioleaching and shortening the total duration of the leaching

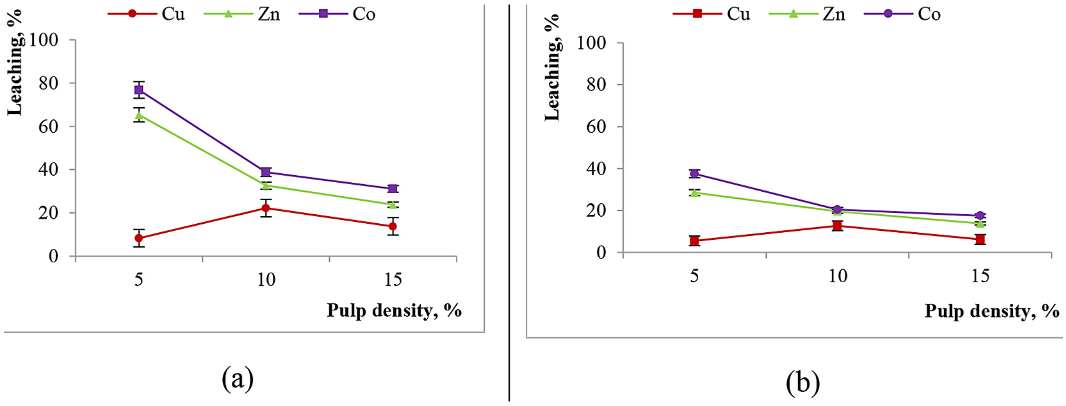


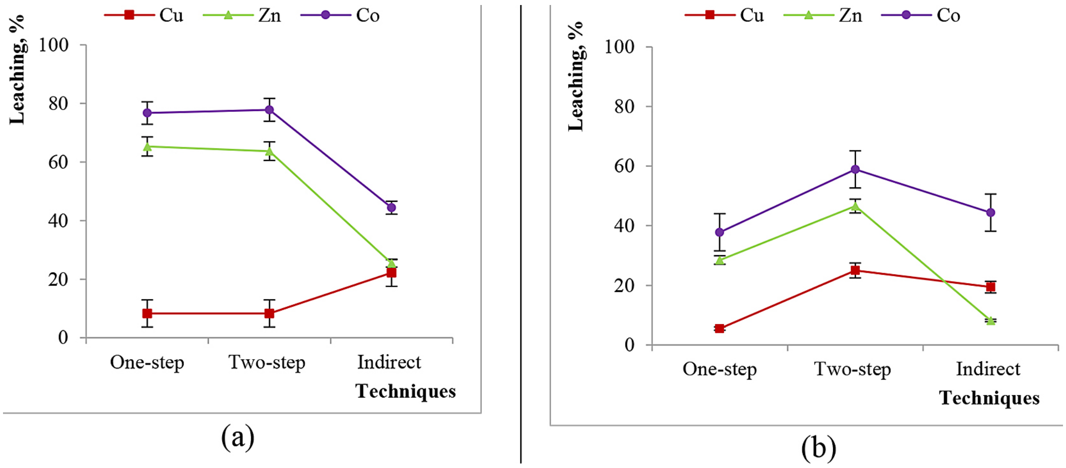
Figure 3. Effect of pulp density on the non-ferrous metals recovery by direct one-step bioleaching with *A. niger* (a), and *P. ochrochloron* (b)

process. After a week of preliminary cultivation, copper slag was added to the spent medium containing the fungal biomass, marking the transition from the first to the second step of the direct bioleaching technique. The response of the fungal biomass to the organisation of the copper slag bioleaching process can vary, affecting the recovery of non-ferrous metals. Some researchers have reported that excess fungal biomass at the start of an experiment can lead to reduced organic acid synthesis due to various factors, including sucrose and NH_4^+ concentration and aeration rate [50]. For example, the results of non-ferrous metal bioleaching after the second step using *A. niger* are comparable to those of the one-step bioleaching technique at 5% pulp density (see Figure 4). This indicates that *A. niger* adapts quickly and easily to copper concentrations in the pulp, enabling it to sequester copper from cobalt and zinc, leached easily under these conditions like as in the one-step leaching technique.

Based on the results of the reduction in the weight of the copper slag sample determined at the end of the direct two-step bioleaching technique, the morphology of *A. niger* biomass and its interaction with copper slag particles formed during the second step, as well as the value of fungal biomass, reveal that the chemical composition and mineralogy of the copper slag sample and mineral particles have no adverse effect on the growth and activity of *A. niger*, in contrast to the one-step leaching technique at 5% pulp density.

The two-step technique of copper slag bioleaching with *P. ochrochloron* has a positive effect on the recovery of non-ferrous metals, as evidenced by the results compared to those of the one-step technique (Figure 4). For example, copper recovery increases almost fivefold, reaching 25% after three weeks of cultivation. Zinc (46.6%) and cobalt (58.9%) recovery also improves when bioleaching starts with more fungal biomass at the beginning of the second stage of the experiment. It indicates that the copper slag particles adversely affect *P. ochrochloron*, hindering its growth and activity during the first 7–10 days of the one-step bioleaching technique, resulting in a lower rate of bioleaching of non-ferrous metals. This negative impact diminishes when the experiment begins with greater fungal biomass, promoting the even distribution of copper slag particles on its surface and facilitating their interaction and the formation of small grains during bioleaching. Therefore, the results of the two-step technique for copper slag bioleaching with *P. ochrochloron* demonstrate the primary role of direct contact between raw material particles and microbial cells, as well as the mechanisms initiated in microbial metabolism and non-ferrous metal bioleaching.

The direct techniques of copper slag bioleaching in this study were carried out in the presence of a single fungal species. Using consortia consisting of two or more fungal species in raw materials bioleaching will not improve the results for the base metals leaching. There are several reasons which would determine the result proposed above. Firstly, the *A. niger* and *P. ochrochloron* occupy the same ecological niche, leading to a competitive interspecies relationship between them when both species are cultivated together. Consequently, the faster-growing species will outcompete and restrict the growth of slower-growing species over time. Secondly, the role of each fungal species will depend on its adaptation to the leached ions and their concentrations in the



(a)

(b)

Figure 4. Effect of bioleaching techniques on the non-ferrous metals recovery from copper slag with *A. niger* (a), and *P. ochrochloron* (b) at 5% pulp density

pregnant leach solutions. Comparing the results from the cultivation of both fungal species, *A. niger* is a faster-growing species that exhibits adaptation to higher concentrations of non-ferrous metals, including iron, manganese, and aluminium, compared to *P. ochrochloron*. Therefore, it is realistic to expect that *A. niger* will be dominant and will have a primary role in non-ferrous metals recovery from copper slag if their bioleaching is initiated with a consortium consisting of both fungal species.

Direct techniques of copper slag bioleaching with fungi at a lower pulp density of 5% require 28 days, which is not realistic for the application of that processing approach on an industrial scale. However, indirect bioleaching of copper slag with spent medium, obtained from cultivation of relevant fungal species (spent medium of *A. niger*, in that case), possesses features (such as variation in pulp density, extra acidity addition, conducting the leaching at higher temperature), which significantly increase the rate of base metals leaching and reduce the copper slag processing time. Therefore, the indirect technique of raw materials bioleaching is a more realistic approach for the recovery of base metals from copper slags in a sustainable way on an industrial scale.

Indirect technique for bioleaching of non-ferrous metals from copper slag

Indirect leaching is carried out using the spent medium obtained after cultivating the relevant fungal species for one week. This medium contains metabolites produced during the growth process. The main difference between the spent medium of *A. niger* and *P. ochrochloron* lies in the concentration of citric and oxalic acids and the total amount of hydrogen ions released into the solution due to the dissociation of the carboxyl groups of the abovementioned organic acids. The spent medium of *A. niger* contains higher levels of citric acid than oxalic acid, resulting in a highly acidic pH of 2.58 and a total acidity value of almost 1.0 (Table 2). Therefore, acidolysis is the primary mechanism for leaching non-ferrous metals from copper slag, as described in reactions 4 and 5. Results regarding the leaching of non-ferrous metals from copper slag using the spent medium of the two fungal species are similar (Figure 4) and higher than those of chemical leaching with sulfuric acid at a similar pH. For example, the final pH measured at 5% pulp density was 4.39 and 5.29 for the spent medium of *A. niger* and *P. ochrochloron*, respectively. Better leaching of non-ferrous metals was achieved with the *A. niger* solution, yielding 22.2%, 25.4%, and 44.4% for copper, zinc, and cobalt, respectively. The concentration of oxalic acid in both types of spent medium is limited during the indirect leaching experiment, which restricts the formation of copper oxalate. It explains why copper leaching improves by almost a factor of three when the indirect technique is applied compared to direct leaching techniques. Despite the final pH value of 5.29, the *P. ochrochloron* spent medium leaches 19.4% of copper. It shows that, via the formation of stable organometallic complexes with a suitable net charge between base metal cations and organic anions, complexolysis acts as an auxiliary mechanism for non-ferrous leaching and accelerates the acidolysis effect on non-ferrous metal leaching, especially at slightly acidic pH.

The main advantage of indirect leaching of raw materials is that it enables the distinction of the relative portions of the different mechanisms involved in non-ferrous metals, due to metabolite secretion by relevant microbial species or consortia, and due to the intimate interaction between microbial cells and mineral particles. The results of the experiments reveal that approximately half of the zinc and cobalt leached by the direct techniques is due to the concentration and ratio of secreted organic acids by the relevant fungal species in the spent medium. The remaining non-ferrous metals are leached due to the long-term interaction between fungal hyphae and copper slag particles.

Bioleaching using heterotrophs is an efficient technique for processing raw materials that contain high levels of oxides and silicates. However, there are some limitations to direct techniques, such as the total content of organic acids (secreted in the solution due to microbial activity) and the lower temperatures at which bioleaching is carried out. Indirect bioleaching using spent media can overcome these limitations by conducting the leaching at temperatures above 45 °C and/or supplementing the leaching solution with additional acid [4, 28, 38].

The stability of the soluble complexes formed between non-ferrous metals and ligands (citric anion, mainly) derived from organic acids (citric acid, respectively) produced by fungi over time was not investigated in this study. The reason is that when the bioleaching process of non-ferrous metals from raw material is optimised, the following steps in the raw material flowsheet are solid-liquid separation (usually by filtration) and the processing of the pregnant leach solution through sequential separation of iron and non-ferrous metals, followed by their transformation into value-added products [5] Therefore, the long-term storage of pregnant leach solutions, obtained from raw materials bioleaching with fungi, will be avoided, and questions about the stability of the formed complexes, which they contain, will be insignificant.

The economic viability of each technology for raw materials processing depends on several factors, including the efficiency of valuable compound recovery, the value of operational expenses, and the price of the relevant valuable compounds on the international market. At this stage, the economic viability of fungal bioleaching is a more expensive approach for copper slag processing compared to classical chemical leaching with sulfuric acid at pH 3.0. The economic viability study conducted by Que et al. [15] revealed that the estimated cost of recovering rare elements from red mud by fungal bioleaching is twice as high as the respective cost of chemical leaching with sulfuric acid. The use of sucrose, a costly compound, in nutrient media for *Aspergillus niger* cultivation, as well as the requirement for bioreactor sterilisation before the start of cultivation/bioleaching operations, are the primary reasons for the higher operational expenses. Using rich-in-cellulose organic wastes, instead of sucrose, is an alternative that can decrease operational expenses and improve the economic viability of fungal bioleaching as a processing approach. However, the effect of changes in the nutrient medium content on the growth of fungi and the corresponding biological extraction of non-ferrous metals from copper slag using various techniques (direct and indirect) needs to be re-examined in the laboratory.

Analysis of copper slag leaching residues

Reduction of the copper slag weight

The visible change in colour of the solution and fungal biomass during bioleaching, turning bright green in experiments with *P. ochrochloron* and dark grassy green in experiments with *A. niger*, indicates substantial co-leaching of iron during base metal recovery from a copper slag sample. Therefore, the reduction in the weight of the copper slag, as determined after the relevant leaching period with the respective fungal species, is a characteristic used to evaluate the complex changes in the chemical composition and mineralogy of the samples undergoing fungal leaching. This analysis is performed after the copper slag residues have been washed with distilled water at the end of the relevant leaching period, dried to a constant weight and ashed at 500 °C. Thus, copper slag bioleaching using the direct one-step technique at 5% pulp density with *A. niger* results in a 35.2% reduction in sample weight after four weeks (Figure 5).

During this period, the total content of iron and silicon decreased by 64.8% and 25.2%, respectively, which indicates substantial co-leaching of these two structural elements. These values are approximately twice as low as those obtained by chemically leaching both elements with sulfuric acid at pH 3.0. It suggests that, due to the encapsulation of non-ferrous metals in the crystal structure of primary minerals, their recovery by fungal bioleaching requires significant co-leaching of iron and silicon from copper slag. Copper slag weight reduction due to *P. ochrochloron* bioleaching on nutrient media with an identical composition and under the same experimental conditions is 7.1%. Co-leaching values of 2.3% and 11.6% for iron and silicon, respectively, were observed. These results demonstrate the greater bioleaching potential of the *A. niger* strain for non-ferrous metals in copper slag compared to the *P. ochrochloron* strain due to its higher growth rate and organic acid yield values. These results are consistent with those of other studies, which suggest that *A. niger* is the most effective strain for the fungal bioleaching of base and rare elements from various types of raw material.

The primary factors influencing iron bioleaching from raw materials are the pH at which the process is carried out and the variety and content of iron-containing minerals. Therefore, iron recovery due to copper slag bioleaching with acidophilic, chemolithotrophic bacteria growing at an acidic pH is above 40%, due to the excess hydrogen ions consumed in the acidolysis process to maintain a pH below 3.0 [24, 25]. Another option for bioleaching copper slag heterotrophically is to use silicate-solubilising bacteria (*Bacillus* spp., *Pseudomonas* spp.) and NH_4-producing bacteria (*Sporosarcina* spp.), which grow at a pH between 7 and 10.5. However, iron recovery is typically less than 1% due to the sparing solubility of iron in aerobic conditions and at neutral to alkaline pH [24], while silicon leaching is significantly enhanced.

XRD analysis

XRD analysis of copper slag reveals phase transformations that occur before and after applying different bioleaching techniques using *Aspergillus niger* or its metabolites at a 5% pulp density. Figure 6 shows the results of XRD analysis of copper slag samples after two, three and four weeks of direct bioleaching with *A. niger* at a temperature of 25 °C.

XRD analysis of the raw sample revealed that the copper slag consisted of compounds with phase structures of protomangano-ferro-anthophyllite, fayalite, and an unidentified type of pyroxene. Protomangano-ferro-anthophyllite ($H_2Fe_{4.48}Mg_{1.08}Mn_{1.44}Si_8O_{24}$) was the most susceptible to bioleaching of the copper slag compounds, with a relative content of 30.0%. The XRD pattern indicates that the mineral peaks are distributed uniformly between 10°, 20°, 30°, 35°, 40°, 50°, and 70° 2θ. Direct one-step bioleaching of the sample with *A. niger* over a period of one month reduces the relative mineral content to 10.0%, with two distinct stages in the process. Protomangano-ferro-anthophyllite exhibited accelerated leaching during the initial three weeks of the experiment, resulting in a 50.0% reduction in its relative mineral content (see Table 3). During this period, the iron and silicon concentrations

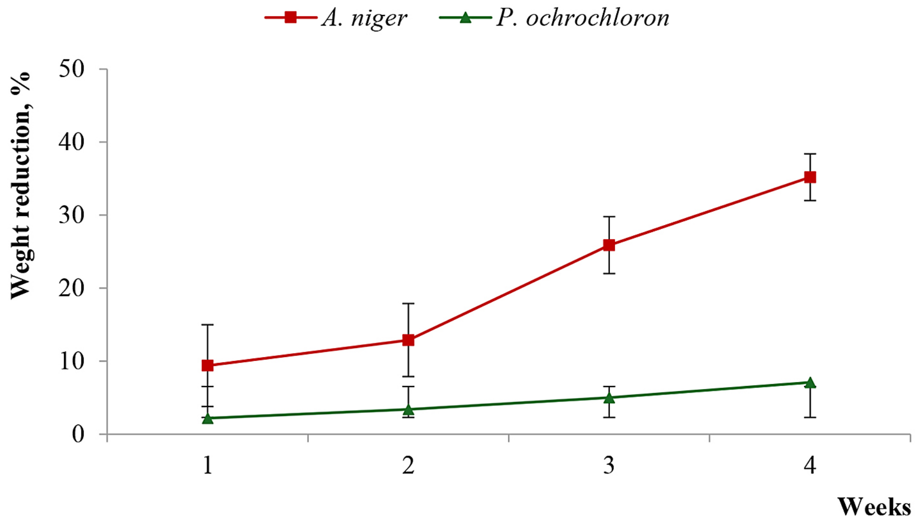


Figure 5. Reduction of copper slag weight after 28 days of bioleaching with *A. niger* and *P. ochrochloron* at 5% pulp density by direct one-step technique

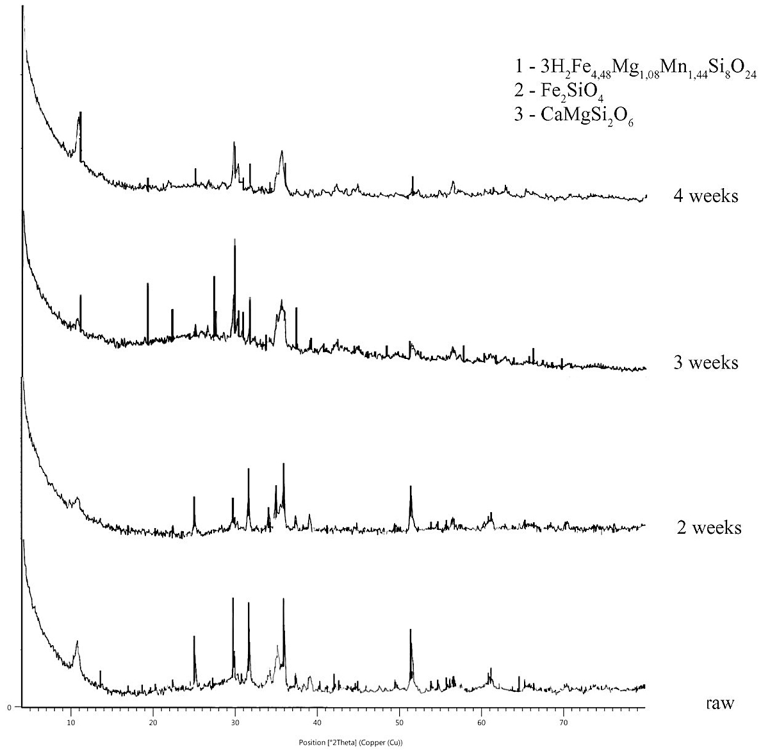


Figure 6. XRD pattern of copper slag before and during its bioleaching with *A. niger*

in the pregnant leach solution were 1980 mg/L and 1760 mg/L, respectively, indicating 6.3% and 14.7% leaching of the two-copper slag structural elements. In the final week of the experiment, protomangano-ferroanthophyllite leached more slowly, and its relative content decreased by a further 16.7%. The XRD analysis conducted after one month of copper slag bioleaching has revealed that the protomangano-ferro-anthophyllite bioleaching has vacated the area, lying between 30° and 70°. Fayalite (Fe_2SiO_4) has been identified as the second most abundant mineral in slag, with a relative content of 50.0%, occupying the 15–35 and 72–78°2θ copper positions. During the initial bioleaching phase, characterised by elevated protomangano-ferroanthophyllite content in the copper slag and a pH above 4.0, fayalite exhibits resistance to both direct and indirect fungal leaching mechanisms. The relative content of the substance under investigation increased to 55.0% of the sample. However, a marked increase in the leaching rate was observed during the final week of the experiment, accompanied by a 40.0% decrease in its relative content. The XRD pattern indicates that the mineral intensity is most pronounced at the 28, 35, 45 and 75 positions.

The third constituent of the raw copper slag is an unidentified type of pyroxene ($Ca_{0.949}Fe_1Na_{0.051}Si_2O_6$), accounting for 20.0% of the composition. The XRD pattern reveals that it occupies zones 31 and 33–35, 48–50 and 72–80. A one-step leaching technique involving a fungal attack by A. niger hyphae has been shown to remove some impurities from the mineral structure. Following a week-long bioleaching process, the mineral was identified as diopside ($CaMgSi_2O_6$) based on its XRD pattern. However, it exhibits resistance to further bioleaching under the conditions that are maintained due to fungal growth and activity. After one month, the relative content of the substance in the copper slag sample increased to 60.0%. The XRD results revealed an increase in the intensity around the 30, 40, 50, 55–65 and 70–80 2θ copper (Cu) positions.

Using the standard chemical leaching tests (TCLP, SPLP) reveals that the raw copper slag sample and the obtained bioleaching residue do not exceed the relevant regulatory limits for non-ferrous metals in solution. However, the disposal of bioleaching residue is not an option for storage due to the domination of particles with sizes less than 50 µm in their particle size distribution characteristics. The lower content of non-ferrous metals and iron (due to the selective leaching of protomangano-ferro-anthophyllite

Table 3. Redistribution of copper slag minerals due to its one-step bioleaching with *A. niger* at 5% pulp density

Parameter	Relative content, %		
	Fayalite	Unnamed pyroxene/ diopside	Protomangano-ferro-anthophyllite
Raw copper slag sample	50	20	30
Direct one-step bioleaching with *A. niger*			
2 weeks	55	23	22
3 weeks	50	35	15
4 weeks	30	60	10

and fayalite), and the higher silicon (due to the accumulation of diopside, resistant under these conditions) are characteristics of copper slag residue that enhance their inclusion in the recipe for the preparation of different cement classes. So, the non-ferrous metals bioleaching from copper slag with fungi will turn it into a zero-waste technology.

Morphological analysis

The morphological analysis of the copper slag raw sample, carried out by SEM, reveals that it consists of small particles with a grainy structure, distributed almost evenly on a base on which medium- and large-size particles are disposed (Figure 7a). These particles are characterised by their regular form, flat surfaces, and sharp edges.

Image b presents the copper slag sample after the end of a month-direct bioleaching with *A. niger*. The copper slag sample was subjected to a thorough washing process, followed by a drying procedure that maintained its constant weight. These steps were undertaken prior to the analysis. The image reveals the presence of short and tiny hyphae distributed on the surface of medium- and large-size particles, orientated parallel to each other and not visible in image a. In similar experiments about direct bioleaching, *A. niger* forms mycelia with dimensions of 2–10 μm in width and up to 300 μm in length [51]. In addition to the secreted metabolites, the mechanical pressure exerted by the fungal mycelium on solid particles has been demonstrated to enhance leaching. Fungal metabolites, constituents of spent medium and the main factor in the indirect leaching process, have been shown to transform the smooth surface of solid particles into a rough surface with pores or holes [50, 52].

Figure 7c and Figure 7d illustrate the copper slag sample following two and four weeks of bioleaching with *A. niger*, respectively. In both cases, the dried samples were ashed prior to SEM analysis in order to reveal the particle's surface from microbial biomass and the marks of its activity to be visible. Following a fortnight of direct bioleaching (Fig. 7c), the indications of *A. niger* activity on the particle's surface are discernible only at higher magnification during SEM analysis, manifesting as miniscule and superficial scratches oriented parallel to each other. It is important to note that these marks are only visible on the surface of medium- and large-size particles. The observed changes in the morphology of the particles are attributed to the midpoint of copper slag bioleaching at a pH of 4.28, which primarily results in the leaching of protomangano-ferroanthophyllite. Following a period of four weeks of direct bioleaching (see Image d), the presence of *A. niger* is readily discernible as furrows on the surface of medium- and large-size particles, even at lower magnification during SEM analysis, as their dimensions are: an average length of 75–100 μm and a width of 3–5μm, respectively. These alterations in the particle morphology are designated as the second phase of copper slag bioleaching when the pH is less than 3.50, and fayalite and the residual amount of protomangano-ferroanthophyllite are leached concurrently. It is not possible to discern marks from *A. niger* activity on the small-size particles of the copper slag residue.

Based on the similarity in chemical composition and mineralogy of the studied copper slag sample with copper slag from other countries, as well as with other types of metallurgical slag, similar behaviour in the biological extraction of the base metals they contain can be expected. Therefore, the results of the biological extraction of non-ferrous metals with fungi from copper slag from Eliseyna can be generalized for this type of industrial waste.

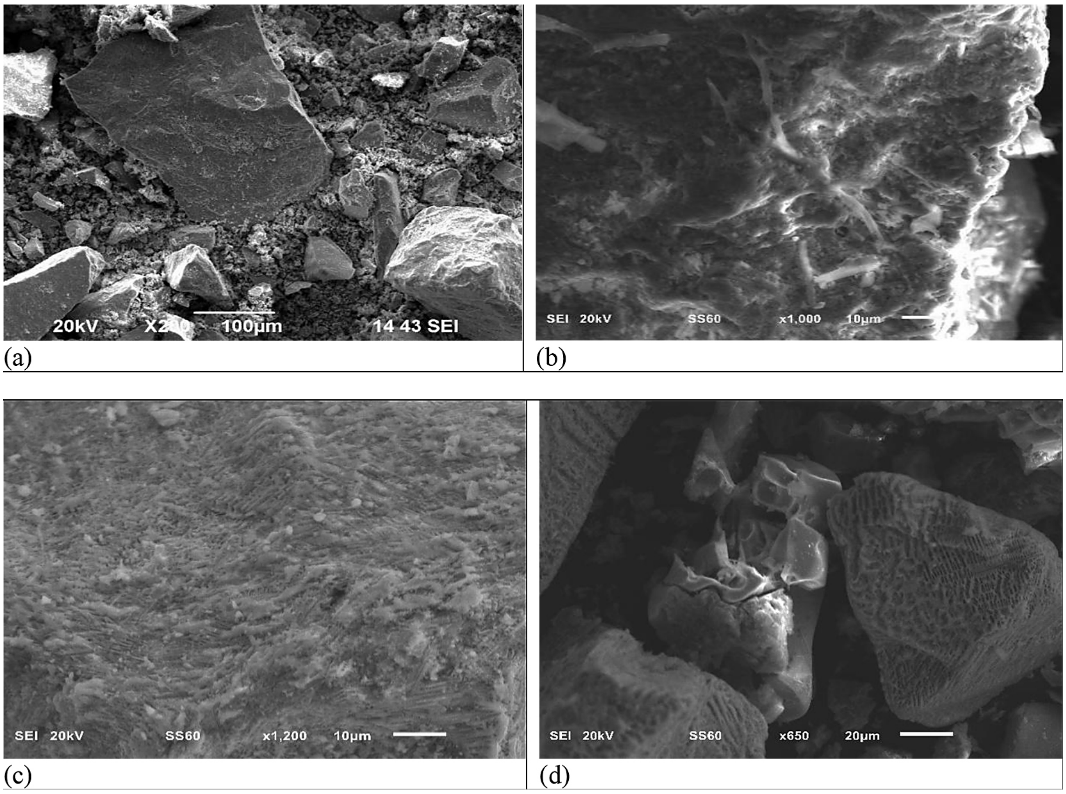


Figure 7. SEM morphology of raw copper slag (a), copper slag with *A. niger* biomass (b), copper slag after two weeks of bioleaching with *A. niger* (c), copper slag after four weeks of bioleaching (d)

CONCLUSIONS

Copper slag from Eliseyna, Bulgaria, was subjected to bioleaching with the fungi *Aspergillus niger* and *Penicillium ochrochloron*, and it was found that fungal activity and their metabolites had a positive effect on the extraction of zinc and cobalt. The values obtained were higher than those obtained by chemical leaching of copper slag with sulfuric acid. *A. niger* showed a higher potential for biological leaching, with extraction almost twice as high as that of *P. ochrochloron*. The optimal conditions for bioleaching of zinc and cobalt from copper slag using *A. niger* were as follows: a pulp density of 5% for all tested bioleaching techniques and a duration of three days for indirect leaching with spent medium, and four weeks for direct techniques (one-step or two-step) in the presence of fungal hyphae. The recovery values of non-ferrous metals obtained by one-step and two-step direct bioleaching with *A. niger* were nearly identical. In both cases, the fungal cells exhibited a protective response to unfavourable changes in the nutrient medium by overproducing oxalic acid and selectively precipitating copper ions, resulting in an insignificant recovery of 8.3%. The process of direct copper slag bioleaching in the presence of *A. niger* has been observed to result in: significant co-leaching of iron and silicon, a phenomenon attributed to the preferential acidolysis of protomangano-ferroanthophyllite and fayalite; and a 35.2% reduction in the weight of copper slag.

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