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Chemical mimicking of bio-assisted aluminium extraction by *Aspergillus niger*'s exometabolites☆

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ABSTRACT

Presence of microorganisms in soils strongly affects mobility of metals. This fact is often excluded when mobile metal fraction in soil is studied using extraction procedures. Thus, the first objective of this paper was to evaluate strain *Aspergillus niger*'s exometabolites contribution on aluminium mobilization. Fungal exudates collected in various time intervals during cultivation were analyzed and used for two-step bio-assisted extraction of alumina and gibbsite. Oxalic, citric and gluconic acids were identified in collected culture media with concentrations up to 68.4, 2.0 and 16.5 mmol L^{-1}, respectively. These exometabolites proved to be the most efficient agents in mobile aluminium fraction extraction with aluminium extraction efficiency reaching almost 2.2%. However, fungal cultivation is time demanding process. Therefore, the second objective was to simplify acquisition of equally efficient extracting agent by chemically mimicking composition of main organic acid components of fungal exudates. This was successfully achieved with organic acids mixture prepared according to medium composition collected on the 12th day of *Aspergillus niger* cultivation. This mixture extracted similar amounts of aluminium from alumina compared to culture medium. The aluminium extraction efficiency from gibbsite by organic acids mixture was lesser than 0.09% which is most likely because of more rigid mineral structure of gibbsite compared to alumina. The prepared organic acid mixture was then successfully applied for aluminium extraction from soil samples and compared to standard single step extraction techniques. This showed there is at least 2.9 times higher content of mobile aluminium fraction in soils than it was previously considered, if contribution of microbial metabolites is considered in extraction procedures. Thus, our contribution highlights the significance of fungal metabolites in aluminium extraction from environmental samples, but it also simplifies the extraction procedure inspired by bio-assisted extraction of aluminium by common soil fungus *A. niger*.

1. Introduction

The carboxylic organic acids, which occur in soils and sediments naturally as microbial or plant exudates (Huang et al., 2016), represent minor fraction of dissolved organic carbon in soils, usually less than 3% (Van Hees et al., 2002; Van Hees et al., 2000). However, their impact on metal biogeochemistry is extremely significant. Low molecular weight organic acids induce changes in the chemical, physical, and biological characteristics of soils as they are involved in mobilizing major and trace elements necessary for organism nutrition (Mimmo et al., 2014; Terzano et al., 2015). Hence, their presence affects the mobile and labile fractions of metals in the environment. Besides low molecular weight organic compounds, the content of mobile fraction of potentially toxic metals is also affected by presence of high molecular weight organic matter and soil pH. While the acidification of soils leads to elimination of the exchangeable sites occupied by acidic cations, including aluminium, and increases the concentrations of dissolved metal in the extractable fraction (Eimil-Fraga et al., 2015; Walna et al., 2005), high content of organic matter in soil generally decreases dissolved metals' activity in surface soils (Wesselink et al., 1996), although it may results in metal accumulation in deeper soil layers (Dijkstra and Fitzhugh, 2003).

Various procedures were applied to estimate mobile metals in soils and sediments, including the diffusive gradients in thin films (Hlodák et al., 2015a; Zhang et al., 2001) and single step or sequential extraction procedures (Gitet et al., 2013; Hlodák et al.,

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2015b; Kubová et al., 2008; Shan et al., 2003). In extraction procedures, the exchangeable and acid soluble metal fraction from soils and sediments exhibits mobility commensurate to natural environment (Bondareva et al., 2014). According to original and modified BCR protocol (Rauret et al., 1999) acetic acid is applied to assess most mobile, acid soluble/exchangeable fraction of metals in the environment. Ecological and ecotoxicological effects of metals and metalloids mostly relate to such fraction as this is readily mobilized and potentially bioavailable (Hlodák et al., 2016). The metals bound to iron and manganese oxides/hydroxides or organic matter are generally less accessible for organisms (Salomons, 1995). However, low molecular mass carboxylic organic acids are used extensively to assess operationally defined potentially mobile and bioavailable phases (Li et al., 2014; Ptak and McBride, 2015; Vítková et al., 2015; Xiao et al., 2015).

It is important to note here that given concentrations of organic acids applied for mobile metal fraction extraction, such as rhizosphere-based extraction method (Feng et al., 2005), do not reflect actual organic acid production capacity of organism (Li et al., 2003). Therefore, we suggest that application of randomly selected organic acids for mobile or bioavailable metal fraction evaluation is ambiguous. The research on organic acid quality and quantity produced by organisms is necessary before they are actually applied in extraction procedures.

Furthermore, literature is omitting significance of microbial contribution on organic acid production in soils and sediments. Fungal consortia are capable of producing significant amounts of organic acids as shown in various studies (Scervino et al., 2010; Urík et al., 2015). This naturally occurring process may also contribute to natural mobilization of the most environmentally hazardous metals in soils, sediments and solid wastes, including aluminium (Vachon et al., 1994). It is necessary to highlight here that fungi excrete anions of organic acids which have exceptional metal chelating properties but do not contribute to acidification of extracellular environment. However, it is well known that fungi are capable of acidifying their surrounding environment by their metabolic activity (Gadd et al., 2012; Urík et al., 2014a). Acidification during fungal growth depends on membrane-located ATP-driven proton pump. This ion-translocating enzyme is responsible for maintaining the electrochemical proton gradient necessary for nutrient uptake (Manavathu et al., 1999).

Microbial organic acid excretion and acidification have also significant impact on metal mobilization in fungal microenvironment and bioavailability of hazardous substances and their further transfer to other organisms. This may even occur in the environments where major environmental hazards were considered stable and general extraction techniques indicated low concentrations of mobile toxic metal fractions. This highlights necessity to reevaluate significance of microbial exometabolites in toxic metal mobilization, especially in case of aluminium which mobility and toxicity is significantly affected by soil concentration of low molecular weight organic compounds with chelating properties, as well as by soil pH (Dijkstra and Fitzhugh, 2003; Van Hees et al., 2000). This underlines importance of pH stratification and microbial organic exometabolites' concentrations in the closest fungal environment when evaluating mobile metal fractions in environmental samples. Consequently, filamentous fungi have been applied for aluminium extraction from various waste materials in biohydrometallurgy (Amiri et al., 2012; Kolenčík et al., 2013; Santhiya and Ting, 2006).

This inspired our study to chemically mimic the bio-assisted metal extraction as an adequate extraction procedure for mobile or bioavailable metal fraction analysis in the environmental samples. For this reason, we objectively selected fungus *Aspergillus niger*, the most common soil fungus, as a model biological extractant. In order to evaluate chemical extraction procedure which efficiently mimics bio-assisted extraction, we have selected alumina (Al_2O_3) as simple model substrate for mobile aluminium fraction determination, and gibbsite as the natural occurring mineral of aluminium hydroxide. The decision to choose aluminium as metal representative was affected by two factors. Firstly, aluminium mobility in the environment is strongly affected by substrate acidity and presence of complexing agents – both affected by fungal presence, and secondly a lot of effort has been given to evaluate aluminium extraction in previous studies (Matúš et al., 2006) allowing us to directly compare the bio-based extraction to other single step extraction techniques.

2. Materials and methods

2.1. Inoculum preparation

Fungal strain *Aspergillus niger* CBS 140837 was originated from mercury contaminated soil from Slovakia (Urík et al., 2014b). Fungal conidia were harvested from the 7-day old colonies on the Sabouraud dextrose agar plates (HiMedia, India) by washing the agar surface with sterile deionized water. The spore suspension, diluted to desired concentration (approximately 10^6 mL^{-1}), was used as an inoculum for further experiments.

2.2. Two-step bio-assisted extraction of aluminium

Two-step bio-assisted extraction experiments were carried out using 250 mL Erlenmeyer flasks with 50 mL of liquid Sabouraud dextrose broth medium (HiMedia, India) with pH 5.6. The medium was inoculated with 50 μL of *Aspergillus niger* spore suspension. The culture media with fungal exudates were collected on 3rd, 6th, 9th, 12th, 15th and 19th cultivation day, filtered through a 0.45 μm pore size cellulose membrane filter (Membrane Solutions LCC, China) and the pH of culture medium was recorded using electrode HI 1230B (Hanna Instruments, USA). The concentration of organic acids of fungal origin in culture medium was analyzed by capillary isotachophoresis. The 50 mL volumes of collected cell-free culture media, which pH were titrated to values in range from 2.5 to 7.5 or remained unchanged, were then used as extracting agent for 24-h static or dynamic (120 rpm) extraction of 0.1 g alumina or 0.153 g of gibbsite in dark at 25 °C. After extraction procedure, the medium was filtered through 0.45 μm pore size membrane filter and total aluminium was determined in filtrates by inductively coupled plasma optical emission spectrometry (ICP-OES).

2.3. Chemical extraction

Two series of 50 mL solutions of either 0.01 $mol.L^{-1}$ NaCl or mixtures of citric ($C_6H_8O_7 \cdot H_2O$; Centralchem, Slovakia), oxalic ($C_2H_2O_4 \cdot 2H_2O$; Centralchem, Slovakia) acids and D-gluconic acid sodium salt ($C_6H_{11}NaO_7$; Sigma-Aldrich, Germany) adjusted to pH 1.45, 1.53 and 2.72 using 1 $mol.L^{-1}$ HCl were prepared according to respective composition of culture media collected for two-step bio-assisted extraction. These organic acid mixtures labeled A, B and C were adjusted according to organic acid composition of culture medium on 3rd, 12th and 19th cultivation day, respectively, as indicated in Table 1. The selected days are showing the most significant differences in composition of three most representative organic acids. Additionally, the organic acid mixtures were prepared and adjusted to pH 2.5, 3.5 and 4.5. All prepared mixtures were then used for 24-h dynamic extraction of 0.1 g alumina or 0.153 g gibbsite at 25 °C in dark. The different weights of mineral phases were applied to achieve similar content of aluminium in these substrates. Thereafter the suspensions with alumina or gibbsite were filtered through 0.45 μm pore size membrane filter

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