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Phanerochaete Chrysosporium Involvement In Medium pH Alteration And Speciation Of Cu At Goethite Surface

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ABSTRACT

Batch experiments in the aerobic nutrient medium indicated that *P.chrysosporium* was involved in medium pH alteration and speciation of Cu at goethite surface. It was observed that pH alteration induced by *P.chrysosporium* was mineral dependant. The interaction between *P.chrysosporium* and goethite might shift the equilibrium to releasing more metal into the aqueous phase due to the decrease of pH in medium, sequestration of Cu by mycelium and formation of soluble organic Cu complex. The conversion of weakly bound Cu to aqueous Cu at the first 96 h of incubation was observed while strongly bound Cu remained nearly constant during the whole experimental period. The re-immobilization of Cu at 192 h was occurred in the presence of *P.chrysosporium*, which could be explained as being the result of both Cu precipitation with the increased pH and complexing of Cu by carboxyl, phenolic and carbonyl groups of the organic phase. In conclusion, *P.chrysosporium*-mineral interaction can play a key role in the cycling of trace elements in natural systems. A better understanding of fungal-mineral interactions is an urgent need to bioremediate the contaminated soil with fungi.

KEYWORDS

Fungi;
Heavy metal;
Cu;
Goethite.

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INTRODUCTION

Iron oxides are ubiquitous in natural sediments, soils and they play an important role in a number of environmental processes, including nutrient cycling, plant growth, contaminant migration. Over the past two decades, dissimilatory anaerobic dissolution of ferric iron minerals had been documented for a wide range of microorganisms in a broad range of environments[1-5] and had become recognized as an important factor on the heavy metal immobilization or mobilization on the surface of various iron oxides[6,7]. Surprisingly little, however, had been published with respect to the effect of aerobic process of microorganisms on the stability of minerals and the role of fungi-mineral interaction on cycling and speciation of metals that was known to interact strongly with iron oxide surface. In the soil, fungi comprised the largest pool of biomass (including other microbes and invertebrates), especially in sites contaminated with toxic metals and radionuclides[8,9]. It was shown that metal inputs increased ergosterol and decreased bacterial-fungal PLFA ratios in most soils indicating the comparative increase of fungal biomass compared to bacteria in metal-contaminated soils[10]. This large biomass combined with their filamentous explorative growth habit and high-surface-area-to–mass-ratio, ensured that fungus-mineral interactions were an integral component of environmental cycling processes[8,11].

Fungi could solubilize minerals and metal compounds through acidolysis, complexolysis, redoxolysis and mycelial metal accumulation[12]. Fungi biomass could provide a metal sink, either by metal biosorption or intracellular accumulation[8,9,13]. Organic acids formed by the metabolic activity of fungi can lower the pH of the system to values that interfere with the electrostatic forces that hold heavy metals on the surface of iron or manganese oxide minerals. Displacement of cations by hydrogen ions may lead to the solubilization of the surface-associated metal. In some cases, the organic metabolites also serve as complexing agents that can form soluble metal-ligand complexes. The charged functional groups of cell surface can serve as nucleation sites for deposition of various metal-bearing precipitates. That is to say, in the heavy metal contaminated soil, fungi also have important biogeochemical roles and the effect of fungi-mineral interaction on cycling and speciation of metals was expected to be complex and should not be neglected. Shi et al (2004) reported that white rot fungus *Phanerochaete chrysosporium*, improved the mobilization of Cu and Zn in the contaminated soil[14]. But the mechanism was not clear.

In this paper, we elected to test the role of fungi-goethite interaction with white rot fungus *Phanerochaete-chrysosporium*, an organism widely distributed in soils and known as predominant degraders of lignin. Copper, as a common pollutant metal and an essential metal for the growth of living organisms, was considered in our initial investigation. The object of this study was to observe the pH alteration, Cu desorption and speciation process as the result of the interaction between *P.chrysosporium* and goethite under aerobic condition. The results will be beneficial to further understanding of the important role that fungi play in the biogeochemical cycling of elements.

MATERIALS AND METHODS

Microorganism

P.chrysosporium, kindly provided by Dr. Kun Wu (Henan University), was maintained at 4°C on 2% malt agar slants from which it was transferred to potato extract plates (20g agar l^{-1}, 200g potato extract l^{-1}, 20g glucose l^{-1}). The plates were incubated at 37°C for 4 days. The mycelium was suspended in 0.1 M phosphate buffer with the optical density (A_{600}) of 0.4 (corresponding to approximately 6.03 mg dry weight l^{-1}).

Preparation and characterization of goethite

Goethite was formed by adjusting the pH of a 0.4 mol/L solution of $FeCl_3.6H_2O$ to 12 with 4 mol/L NaOH and incubating the suspension at 60°C for 24 h. The oxide was then washed free of salt by dialysis, air-dried, and passed through a 60 mesh sieve. X-ray diffraction (XRD) was used to confirm the identity of goethite (Figure 1). Powder XRD patterns were obtained with a Rigaku D/max 2550 P system, using CuKa radiation with a variable divergent slit and a solid-state detector. The routine power was

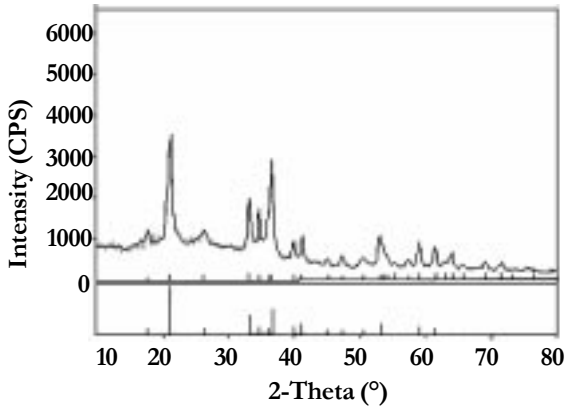

Figure 1: X-ray diffraction (XRD) of goethite

12000 W (40KV, 300mA). Low background quartz XRD slides were used.

Culture experiments

To prepare the experimental slurry, 0.1 g of sieved goethite was added to acid-washed, glass bottles containing 25 ml of phosphate buffer (4.8 g of K_2HPO_4, 1.2 g of KH_2PO_4, and 1000 ml of distilled H_2O) or nutrient medium (4.8 g of K_2HPO_4, 1.2 g of KH_2PO_4, 10.0 g of glucose, 1 g of $NaNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1000 ml of distilled H_2O). This slurry was autoclaved and subsequently cooled. One milliliter of sterile Cu stock solution (10000 μmol/L) was added to each bottle. The volume was adjusted to 26 ml with an appropriate volume of sterile water, and the solution was allowed to equilibrate for a period of 60 h. Experiments were then initiated by inoculating slurries with 1 ml of *P. chrysosporium* or adding 1 ml of sterile phosphate buffer as non-inoculated control. Initial samples (t_0) were taken from different treatment for subsequent analysis prior to inoculation with *P. chrysosporium*. Both inoculated and non-inoculated bottles were under aerobic sterile condition and incubated horizontally on a shaker table at 25°C in dark.

Sampling and analysis

At pre-determined time periods, one to three bottles per treatment were devastatingly sampled. Bottles were shaken to resuspend any settled solid and slurry aliquot were withdrawn by pipet and used for further analysis.

Total Cu in the cultures was determined by extraction with 6 mol/L HCl for not less than 4 h until all solid were dissolved. Liable extractable Cu, which was weakly adsorbed to iron oxide surface or dissolved in the culture medium, was determined by extraction with 0.5 mol/L HCl for 60 min and measurement of Cu in the supernatant after separated by centrifugation (10000 × g for 10 min). Dissolved Cu was determined by directly centrifuging an aliquot of slurry at 10000 × g for 10 min and filtering. Weakly bound Cu was calculated as the difference between 0.5 mol/L HCl extractable and dissolved Cu. Strongly bound Cu was calculated as the difference between total Cu in the culture and 0.5 mol/L HCl extractable Cu as described in reference[7].

For evaluating the influence of microbial growth on extractability of Cu, an aliquot of slurry was transferred to a microcentrifuge tube and extracted in 0.1 mol/L NaOH for a period of 60 min. The solid was separated by centrifugation (10000 × g for 10 min) and the supernatant was analyzed. Here, sodium hydroxide was chosen as an extractant for three reasons: (1) sodium hydroxide was a suitable extractant for organic substances[15] (2) sodium hydroxide did not extract the ion Cu (3) 0.1 mol/L sodium hydroxide proved to be a suitable extractant for Cd bound to microorganisms[16].

Copper and iron concentrations were determined by an atomic absorption spectrophotometer (Perkin-Elmer 100 AAnalyser). pH in the aqueous phase was determined by Beckman Φ720 pH Meter.

RESULTS AND DISCUSSION

Medium pH alteration

Figure 2 showed the pH alteration in the medium as a result of microbial growth. Medium pH remained consistent with the cell free controls over the first 36 h of incubation, decreased to pH 6.01 over the next 36 h in the presence of goethite and then quickly increased back to the pH value even one unit higher than initial level. Since P. chrysosporium had no apparent effect on the pH alteration in the mineral free control, pH alteration in the medium seemed mineral depended. It was reported that mineral surface properties (e.g. microtopography, surface composition, surface charge and hydrophobicity) play an integral role in microbial attachment and detach-

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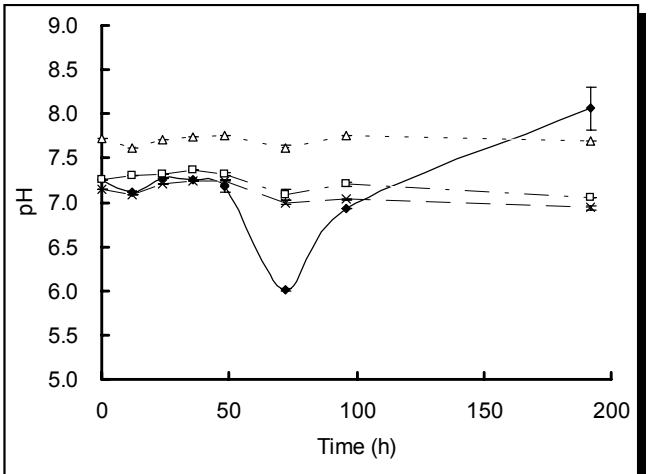

Figure 2: pH alteration in the medium as a result of microbial growth. Inoculation (♦), inoculation in phosphorus buffer (Δ), cell free control (□), and mineral free control (×)

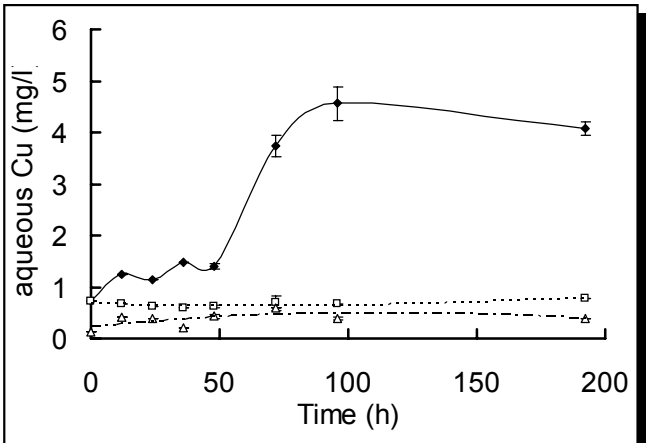

Figure 3: Copper desorption from the surface of goethite. Inoculation (♦), cell free control (□) and inoculation in phosphorous buffer (Δ)

ment processes and biofilm formation[17-20]. Microbial growth, metabolic activity and genetic processes can be strongly influenced by clay[21,22]. The formation of organic matter, e.g. humic acids, can result from a combination of processes that rely on both mineral surfaces and microbial metabolism[23,24]. In this experiment, the observed acidification during the incubation of 48-72 h was probably related with metabolic activity and resulted from (1) the excretion of protons via the proton translocating ATPase (2) the absorption of nutrients in exchange for protons (3) excretion of organic acids (4) carbonic acid formed as result of respiratory CO_2 production[25]. After 72 h, release of OH^- during goethite reduction in the presence of metabolites was probably the cause for the elevated pH. Previous studies of iron(III) oxide reduction by Shewanella alga strain BrY was widely reported[1]. Such pH modifications will lead to a greater degree of cation adsorption and may lead to the precipitation of mineral assemblages. Thus the pH alteration induced by the interaction of goethite and *P. chrysosporium* could play an important role in cycling and speciation of Cu in the environment.

Desorption of Cu from the surface of goethite

With initial Cu concentration of approximately 0.7mg/L in the aqueous-phase, it suggested that during the time period requested to set up the experiment (ca. 60 h), 97% of the added Cu was bound to goethite (Figure 3). In two control experiments (cell free control and inoculation in phosphorous buffer), aqueous phase Cu did not increase after initiation of the experiment, which indicated that a steady-state in adsorption-desorption had been achieved. In inoculated treatments with nutrient medium, however, aqueous Cu increased throughout the duration of the experiment, which suggested the microbial mediated desorption of Cu on the surface of goethite had happened. The highest Cu concentration in aqueous phase was observed at the sampling time of 96 h, whereas the medium pH was 6.93. Since pH alteration was not consistent with the variance of aqueous Cu, we speculated that the excretion of H^+ with the growth of *P.chrysosporium* was not the only reason for the increased Cu desorption on the surface goethite. Metal-binding to the fungi and formation of organic complex Cu might be a potential mechanism for the increased Cu desorption, especially at the medium with higher pH. Burford et al. mentioned that fungal biomass provides a metal sink, either by (1) metal biosorption to biomass (cell walls, pigments and extracellular polysaccharides); (2) intracellular accumulation and sequestration, or (3) precipitation of metal compounds onto hyphae[12]. In addition, fungi, like most heterotrophs, excrete primary and secondary metabolites with chelating properties (e.g. carboxylic acids, amono acids and phenolic compounds)[26,27]. The production of organic acids provides a source of protons for solubilization and metal-chelating anion to

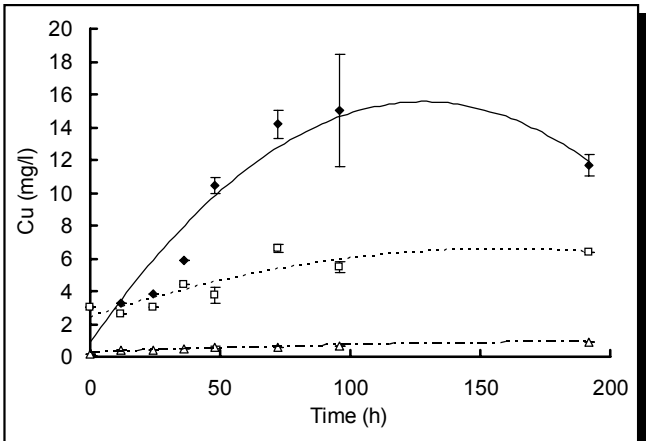

Figure 4: Copper in NaOH extracts as a result of microbial growth. inoculation (♦), cell free control (□) and inoculation in phosphorus (Δ)

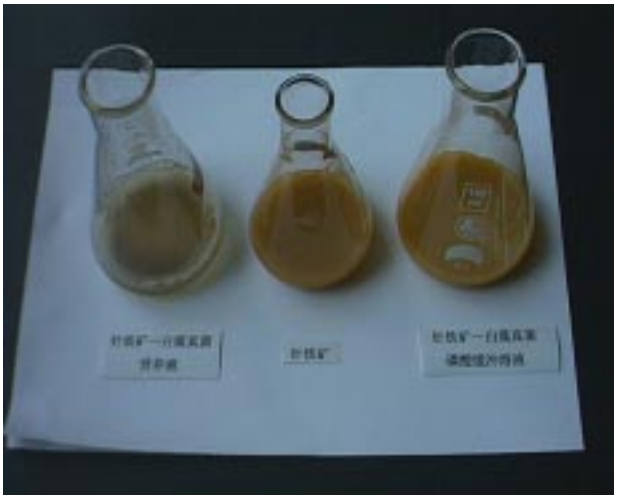

Figure 5: Goethite aggregates after inoculation of *P.chrysosporium* in nutrient medium. A: Inoculation in nutrient; B: Cell free control; C: Inoculation in phosphorous buffer

complex the metal cation.

For evaluating the influence of microbial growth on Cu desorption from iron oxides, an aliquot of slurry from inoculated and cell free control treatments was transferred to a microcentrifuge tube and extracted in 0.1 mol/L sodium hydroxide solution. Figure 4 showed the Cu concentration in 0.1 mol/L NaOH extracts as a result of microbial growth. The nutrient media caused a considerable increase in the amount of Cu extractable with NaOH: 4.65 mg/L in the average of Cu was extracted with NaOH in the goethite suspension with nutrient (cell free control), versus 0.52 mg/L in the average from that in buffer (inoculation in phosphorous buffer). After 48 h of incubation, the growth of *P. chrysosporium* could be observed distinctly only in nutrient medium. The increases in Cu concentration in NaOH extracts were accompanied by the microbial growth, apparently indicating that the fate of Cu was influenced by *P.chrysosporium* in the suspension. As earlier research reported, the intensive activity of microorganisms could cause the production of low molecular matter, such as organic acid, and the formation of aromatic substances, probably extracellular polymers and products of cell lysis[26,27]. Therefore, the NaOH extractable Cu might be include the copper sequestrated by mycelium, water soluble organic complex Cu, and water insoluble organic polymerized Cu. The two former were most likely dissolved in the liquid phase and extracted as aqueous Cu, since no living mycelium was observed in mineral phase by microscope. The latter, however, was likely on the solid phase, since large aggregates were observed in all inoculated treatments (Figure 5). Hence, it could be concluded that with the growth of *P. chrysosporium*, Cu retention on the organic phase was increased and mycelial metal accumulation and complexolysis were probably the main mechanisms for the Cu desorption from the surface of goethite.

Cu speciation at goethite surface

As discussed above, inoculation of *P. chrysosporium* might shift the equilibrium to release Cu into the suspension. Therefore, Cu species on the solid phase must have altered accordingly. Here, we used the term 'weakly bound Cu' referring to the Cu associated with the solid phase that could be extracted by 0.5 mol/L HCl, and 'strongly bound Cu' to the left. Figure 6 showed the effect of microbial growth on Cu speciation at goethite surface. In the cell free control, approximately 97% of the original mass of copper adsorbed on the surface of goethite, 68% of which can be accounted for as weakly bound Cu. Cu species on the goethite surface was not changed during the incubation. In the inoculation treatments, however, approximately 80-97% of the original mass of copper adsorbed on the surface of goethite, 66-75% of which can be accounted for as weakly bound Cu. These findings indicated that in aerobic condi-

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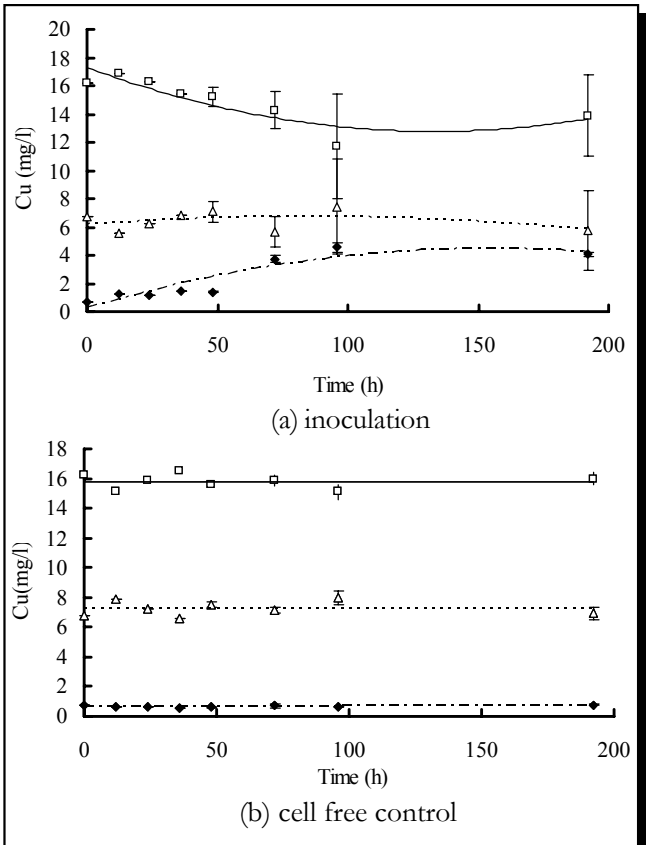

Figure 6: Effect of microbial growth on Cu speciation at goethite surface. Aqueous Cu (♦), weakly bound Cu (□) and strongly bound Cu (Δ)

tion, weakly bound Cu on the solid phase was primary and easy to be desorbed by microbially mediated pH variations and many other changes in solution chemistry (e.g. excretion of organic ligands by fungi). Strongly bound Cu remained nearly constant during the whole experimental period. Thus on the goethite surface, the conversion of weakly bound Cu to aqueous Cu was dominating and, to some extent, probably influenced by mineral-organic matter complexes. The observation of the mineral aggregation by *P. chrysosporium* supported this hypothesis (Figure 5). Tisdall et al suggested that fungal hyphae bring mineral particles and organic materials together to form stable micro-aggregates at least <2 μm, and enmesh micro-aggregates into stable aggregates >50μm diameter[28]. Therefore, the weakly bound Cu with goethite was slightly increased after 192 h of incubation could be explained as being the result of both Cu precipitation with the pH increased back and complexing of Cu by carboxyl, phenolic and carbonyl groups of the organic phase.

CONCLUSIONS

P.chrysosporium was involved in medium pH alteration and speciation of Cu at goethite surface. Due to the medium pH alteration, sequestration of Cu by mycelium and formation of organic Cu complex, Cu activity at the surface of goethite was greatly affected. A better understanding of fungal-mineral interactions is an urgent need to bioremediate the contaminated soil with fungi.

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