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Alteration of the characteristics of extracellular polymeric substances (EPS) extracted from the fungus *Phanerochaete chrysosporium* when exposed to sub-toxic concentrations of nickel (II)

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ABSTRACT

The fungus *Phanerochaete chrysosporium* was incubated at five sub-toxic concentrations of Ni^{2+} (0.5, 1, 5, 10 and 25 mg/L, respectively), and its metal immobilization ability as well as the alteration of some characteristics regarding the extracellular polymeric substances (EPS) were investigated. With the increased Ni^{2+} concentrations in the broth, higher Ni^{2+} amounts were measured in both intact fungal cells (biomass before EPS extraction) and EPS-free biomass (biomass after EPS extraction). The Ni^{2+} immobilization ability of the extracted EPS displayed a similar level at Ni^{2+} concentrations higher than 1 mg/L. The presence of Ni^{2+} in the broth decreased the zeta-potential of the intact biomass and increased cell surface hydrophobicity (CSH). Fourier transform infrared spectroscopy (FT-IR) analyses identified the presence of some functional groups, such as carboxyl, phosphoryl and hydroxyl groups, in the extracted EPS. The high hydrophobicity (> 60%) of the extracted EPS was decreased by the increased Ni^{2+} concentration. The protein (PN) and polysaccharide (PS) content in the EPS was also affected by the increased Ni^{2+} concentration, and the abundance of PN-like molecules ranging from 0.5 kDa to 14 kDa was enriched. However, the fluorescence characteristics and apparent molecular weight (aMW) of the extracted EPS were not affected by the Ni^{2+} concentration. Therefore, one possible defense mechanism developed by the fungus towards Ni^{2+} stress is the adjustment of its EPS composition.

1. Introduction

Nickel is widely used in the metallurgical industry to produce high quality iron-based alloys, and it is also applied as catalyst in the chemical and food industry, or as prime material for the production of paints and batteries (Gikas, 2008). Those manufacturing activities generate a considerable amount of wastewater containing nickel. In aqueous environments, nickel is often present as divalent cation, *i.e.* Ni^{2+}. At micro- or millimolar concentrations, Ni^{2+} may become toxic for microorganisms and hinder their activity. Paraszkiewicz et al. (2009) reported that some hyphal tip cells of the fungus *Curvularia lunata* underwent lysis after 5 h exposure to 0.3 g/L Ni^{2+}. In an activated sludge system, short-term exposure to 1 and 10 mg/L of Ni^{2+} resulted in a sharp decrease of the phosphorus removal efficiency, and long-term exposure to Ni^{2+} (> 60 days) led to a change in the microbial community (Sun et al., 2017). On the other hand, Ni^{2+} is an essential metal that is required as micronutrient at micro-molar concentrations for the microorganisms, and it is also considered as a trace element in the various biological systems (Thanh et al., 2016). For instance, 0.4 mg/L Ni^{2+} was the minimum concentration required in the thermophilic fermentation of glucose and subsequent methane formation (Takashima et al., 2011). In living microbial cells, Ni^{2+} plays a fundamental role in the microbial metabolism, and catalyzes biochemical reactions in the active sites of metallo-enzymes such as urease, hydrogenase, acetyl-CoA synthase, and methyl-CoM reductase (Can et al., 2014; Maroney and Ciurli, 2014).

The production of extracellular polymeric substances (EPS) by microorganisms plays an important role in the microbial tolerance of toxic metals through sequestration of metal ions outside the cells (Clarke et al., 1997). The metal binding ability of EPS thus protects the fungal

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cells against heavy metal inhibition (Sheng et al., 2013). The metal cations immobilized by the EPS can then compensate the negative charges of the cell surface (Urbain et al., 1993), and hence, the presence of divalent metal cations like Ca^{2+} and Mg^{2+} in the growth medium of microorganisms impacts the microbial cell surface properties (Krasowska and Sigler, 2014).

The main organic constituents of EPS include proteins (PN), polysaccharides (PS), uronic acids, and nucleic acids (DNA) (Wingender et al., 1999). They link with each other through electrostatic bonds, hydrogen bonds, van der Waals forces and hydrophobic interactions (Flemming et al., 2016). Limited studies have investigated the hydrophobic features of EPS. The hydrophilic fraction of EPS is more related to the immobilization of metal ions (Vázquez-Juárez et al., 1994), and it binds more metal ions than the hydrophobic fraction (Wei et al., 2017). Therefore, the EPS hydrophobicity also affects the metal removal during biological wastewater treatment.

Bioreactors inoculated with the fungus *Phanerochaete chrysosporium* have advantages when compared with bacterial inoculated reactors (Espinosa-Ortiz et al., 2016; Li et al., 2016). *P. chrysosporium* is a metal-resistant fungus, and is also well-known for its unique ability to secrete ligninolytic enzymes, *i.e.* lignin peroxidase, manganese peroxidase, and laccase, by which a wide range of organic pollutants such as 2,4-dichlorophenol (Chen et al., 2011) and sulfamethoxazole (Guo et al., 2014) can be degraded. Trace amounts of divalent metal cations like Cu^{2+} or Mn^{2+} in the fungal growth medium facilitate the production of ligninolytic enzymes by *P. chrysosporium*, and affect the fungal pelletization and biodegradation processes (Baldrian, 2003). Therefore, the aim of this study was to investigate the effect of trace metals (*i.e.* Ni^{2+}) on the cell surface characteristics (*i.e.* zeta-potential, cell surface hydrophobicity) of *P. chrysosporium*. In addition, since the EPS determine most cell surface properties of the fungus, and also considering that the growth rate of *P. chrysosporium* is inhibited by the presence of 50 mg/L of Ni^{2+} (Falih, 1997), the alteration of EPS characteristics by the fungus when exposed to sub-toxic Ni^{2+} concentrations (< 50 mg/L) during its growth was investigated in this study.

2. Materials and methods

2.1. Fungal pelletization

The fungus *P. chrysosporium* MTCC187 was provided by the Institute of Microbial Technology, Chandigarh (India), and it was continuously maintained in malt agar plates. The fungal spore suspension was prepared by scraping the culture grown on an agar plate for 3 d at 37 °C and transferring it into 50 mL sterile distilled water. The subsequent fungal pelletization process was carried out in 100 mL liquid medium with 10% (*v/v*) inoculum of the fungal suspension (equivalent to about 1.78 g fungal spores (dry weight) in 1 L nutrient broth).

The original nutrient broth contained: 10 g/L glucose, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L NH_4Cl, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.001 g/L thiamine and 5 mL/L trace elements solution (3 g/L $MgSO_4$, 0.5 g/L $MnSO_4$, 1 g/L NaCl, 0.1 g/L $FeSO_4{\cdot}7H_2O$, 0.1 g/L $CoCl_2$, 0.01 g/L $AlK(SO_4)_2{\cdot}12H_2O$, 0.01 g/L $Na_2MoO_4{\cdot}2H_2O$, 1.5 g/L Nitrilotriacetate ($C_6H_9NO_6$), 0.1 g/L $ZnSO_4{\cdot}7H_2O$, 0.1 g/L $CuSO_4$, 0.01 g/L H_3BO_3) (Tien and Kirk, 1988). In order to study the effect of Ni^{2+} on the alteration of fungal EPS, the trace elements solution in the above-mentioned recipe was replaced by an aliquot of a 500 mg/L Ni^{2+} stock solution (prepared from $NiCl_2{\cdot}6H_2O$) to adjust the final Ni^{2+} concentration in the broth at 0.5, 1, 5, 10 or 25 mg/L.

The initial pH of the medium was adjusted to ~4.5 with 1 M HCl. Fungal pellets cultivated in the absence of Ni^{2+} were defined as the control group in this study. The inoculated broth with and without Ni^{2+} addition was incubated at 30 °C on an orbital shaker at 150 rpm for 48 h to produce the fungal pellets. The fungal pellets incubated after 48 h were considered to be in their exponential growth phase (Espinosa-Ortiz et al., 2015). Six replicates of each group were incubated at one time in order to have enough biomass for EPS extraction and further characterization.

After 48 h of incubation, the pH of the liquid medium dropped to 4.0. The fungal pellets were harvested by vacuum filtration, and the dry weight (DW) of the collected biomass was measured (105 °C, 24 h) after washing twice with ultrapure water. The initial Ni^{2+} concentration in the growth medium was measured by flame atomic absorption spectroscopy (FAAS, A Analyst 200, PerkinElmer, USA).

2.2. EPS extraction procedure

Four replicates of the washed fungal pellets from each group were used for the EPS extraction, and each replicate was re-suspended in 15 mL ultrapure water. The remaining two replicates were stored as intact biomass.

The EPS extraction method used in this study was modified from the protocol described by Hou et al. (2013). Briefly, each 15 mL suspension containing the fungal pellets was heated at 60 °C for 10 min in a water bath, and then centrifuged at 12,000 × g at 4 °C for 20 min. Both the supernatant and the residual fungal pellets were collected and stored at -20 °C for further analysis. The supernatant was considered as extracted fungal EPS, whereas the residual fungal pellets were called the EPS-free biomass. The DW of the extracted EPS was measured after 24 h of heating at 105 °C (D'Abzac et al., 2010). The Ni^{2+} concentrations in both intact biomass and extracted EPS were measured by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific Xseries 2, USA) after an acid digestion treatment.

2.3. Zeta-potential measurement

The intact and EPS-free fungal pellets were washed twice with phosphate urea magnesium sulfate (PUM) buffer at pH 7.1 (22.2 g/L K_2HPO_4, 7.26 g/L KH_2PO_4, 1.8 g/L urea (CH_4N_2O), 0.2 g/L $MgSO_4{\cdot}7H_2O$). The washed fungal pellets were then dispersed again in PUM buffer and homogenized using a glass grinder. After allowing it to settle for 30 min at room temperature, the upper suspension which contains an abundance of well-dispersed fungal cells was collected. The collected suspension was diluted by PUM buffer to a concentration equivalent to the absorbance read at 600 nm (Abs_{600}) of 0.4-0.5 using an UV/visible spectrophotometer (Lambda 365, PerkinElmer, USA). 1 mL of the diluted suspension was used to measure the zeta-potential (Zetasizer Nano, ZS90, Malvern, UK). All the analyses were carried out in triplicate.

2.4. Cell surface hydrophobicity (CSH)

The CSH (%) of the intact and the EPS-free fungal cells were determined by microbial attachment to hydrocarbon (MATH) test as described in Rosenberg et al. (1991). The fungal cell suspension was prepared in the same way as described above.

After the preparation, 10 mL of the diluted cell suspension (Abs_{600} = 0.4-0.5) and 5 mL *n*-hexane were added in a 50 mL screw-capped vial. The water and *n*-hexane phases were mixed by a vortex mixer at its maximum speed for 2 min, and phase separation was done for 30 min. 2 mL of the bottom aqueous phase was collected and measured at Abs_{600}. The CSH (%) was calculated according to Eq. (1):

$$\text{CSH}\ (\%) = \frac{\text{Abs}_{\text{initial}} - \text{Abs}_{\text{hexane-treated}}}{\text{Abs}_{\text{initial}}} \times 100\% \quad (1)$$

where, $Abs_{initial}$ and $Abs_{hexane\text{-}treated}$ represent the Abs_{600} values of the dispersed fungal cell suspension before and after adding *n*-hexane, respectively. All the analyses were carried out in triplicate.