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Metarhizium robertsii morphological flexibility during nonylphenol removal

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ABSTRACT

Nonylphenols (NPs) are toxic organic pollutants that cause deleterious effects in various ecosystems. It was formerly documented by us that the cosmopolitan non-ligninolytic fungus *Metarhizium robertsii* is capable of degrading 4-*n*-nonylphenol (4-*n*-NP) with significant efficiency (initial xenobiotic content 50 mg l^{-1}). The present study revealed that *M. robertsii* is able to survive in the presence of high NPs amounts (up to 100 mg l^{-1}), and the observed phenomenon is combined with intensive xenobiotic utilization. Additionally, the formation of small densely packed pellets that predominated during the efficient NP utilization process was observed. Larger pellets emerged in the cultures with "hairy" morphology, which indicated the removal process was complete. The observed changes in pellet morphology resulted from the detrimental influence of NPs on *M. robertsii* as evidenced by viability and cell ultrastructure. For the first time, this study documented the presence of this toxic substrate in the fungal cell wall. It is suggested that the *M. robertsii* morphological changes may serve as a biomarker for the progress of the intense NP utilization process.

1. Introduction

Nonylphenol (NP) is a ubiquitous pollutant, well known for its endocrine system disruption potential because it mimics the natural hormone 17β-estradiol. In the environment, NP originates primarily from nonylphenol ethoxylate biodegradation products, which are commonly used as surfactants, detergents, solubilizers and dispersing agents (Corvini et al., 2006; Soares et al., 2008; De Weert et al., 2011). It has been documented that NP influences various ecosystems because it may be bioaccumulated by living organisms and persists in the environment (Soares et al., 2008; Shan et al., 2010; Nurulnadia et al., 2014).

The presence of NP and its deleterious effects in aquatic ecosystems has been widely discussed (Soares et al., 2008; Lara-Martin et al., 2014; Rocha et al., 2014) and xenobiotic release into terrestrial environments via biosolid application on agricultural land has also been described (Langdon et al., 2011, 2012; Shan et al., 2011). It has been reported that NP concentrations in biosolids produced and applied in the US vary widely (from <5 to >1500 mg kg^{-1}) and its median, minimum and maximum concentrations amount to 261, 2.18 and 1520 mg kg^{-1}, respectively (Kinney et al., 2006). EDCs originating from contaminated biosolids after introduction to the soil are usually degraded by endogenous soil microbiota. Detailed studies have revealed that the half-life values for tNP in biosolid amended soils were 16–23 d, however, it was determined that 15–30% of the initial tNP persisted in soil at 45 d during the study (Brown et al., 2009). tNP occurs in the natural environment as a mixture of a number of isomers. It is known, that different chemical structure of the isomers affects their properties e.g. they exhibit different degradation rates, however, finally none of the isomers persists in the soil after biodegradation (Das and Xia, 2008; Brown et al., 2009). In the present work, tNP and an additional isomer, 4-*n*-NP, that is commonly used in scientific investigations (as previously suggested) were used (Kollmann et al., 2003; Cajthaml et al., 2009; Barlocher et al., 2011).

It was previously documented that several strains of the non-ligninolytic fungus *Metarhizium robertsii* have the ability to degrade 4-*n*-NP, suggesting that this species is involved in NP degradation in the natural habitat (Różalska et al., 2013). *Metarhizium* spp. are common soil fungi. Field experiments determined that the number of *Metarhizium* propagules in soil can reach 10^6 g^{-1} of dry weight (St. Leger et al., 2011). These soil-inhabiting, insect-pathogenic fungi are important in natural ecosystems as a biocontrol agent and they are also extensively used as an

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alternative to chemical insecticides in many biocontrol programs (Fang et al., 2010). The predominant *Metarhizium* species in agricultural or open field habitats is *M. robertsii* (Wyrebek et al., 2011) and the potentially harmful effects of NPs (nonylphenols) originating from biosolids on this species should be considered.

NP utilization by *M. robertsii* was very efficient and was conducted in the presence of a high amount of the xenobiotic, which indicates that this fungus can be used for large scale NP removal (Różalska et al., 2013). Studies concerning *M. robertsii* NP metabolism are being conducted, but it remains necessary to study the fungus' behavior during NP utilization to optimize this process in the future. Moreover, *M. robertsii* is a common non-ligninolytic fungus, with the xenobiotic degradation process conducted inside the cells, and it can also serve as a valuable model for studying detoxification of harmful compounds by fungi.

The aim of this study was to extend the knowledge of the mechanisms involved in efficient NP elimination and detoxification using *M. robertsii* application as a research model.

2. Materials and methods

2.1. Chemicals

4-*n*-NP (4-*n*-nonylphenol) and tNP (technical nonylphenol) (Fluka, Switzerland) were used as substrates. All other chemicals (pure or high purity) were purchased from Sigma–Aldrich, Germany. Reagents used in electron microscopy studies were of high purity grade (Polysciences, USA).

2.2. Microorganism, cultivation conditions and biomass estimation

The strain *M. robertsii* IM 6511 from the collection of the Department of Industrial Microbiology and Biotechnology, University of Łódź, Poland was used in the investigations. The fungus was maintained on ZT agar slants and spores from 14-day-old cultures were used to inoculate 20 ml Sabouraud medium in 100-ml Erlenmeyer flasks, as previously described (Słaba et al., 2009; Różalska et al., 2010). After 24 h incubation, precultures were transferred to sterile, fresh medium at a 1:9 ratio. The cultures were aseptically supplemented with 4-*n*-NP or tNP dissolved in ethanol (stock solution 20 mg ml^{-1}) at initial concentrations of 50 or 100 mg l^{-1}. The control cultures were supplemented with the same amount of ethanol. All samples were incubated at 28 °C on a rotary shaker (150 rpm) in triplicates. At appropriate times, fungal pellets were withdrawn for analyses.

Dry weight biomass was quantified by filtering the entire culture through a pre-weighed Sartorius membrane filter as previously described (Różalska et al., 2010).

2.3. NPs utilization experiments and GC MS/MS analysis

Samples (in triplicates) were extracted twice with 20 ml ethyl acetate after homogenization (MISONIX, England) at 4 °C with a 130 W power input. The extracts were dried using anhydrous sodium sulfate and evaporated under reduced pressure at 40 °C. Ethyl acetate (2 ml) was added to the evaporated samples and 1 ml was transferred to chromatography vials to determine the NP amount using a gas chromatograph, GC MS/MS Agilent 7890, equipped with a 5975 mass selective detector (Agilent, USA). Separations were performed on an Agilent HP 5MS capillary column (30 m × 250 μm × 0.25 μm) as it was described previously (Szewczyk et al., 2014). The injection volume was 2 μl. The inlet was set to a splitless mode with the temperature maintained at 275 °C. Helium was used as the carrier gas. Column temperature parameters were as follows: 60 °C maintained for 2 min, 20 °C min^{-1} to 300 °C and maintained for 8 min. Mass selective detector parameters were set in scan mode as follows: ms source 250 °C, ms quad 200 °C. The quantitative analyses were performed using standard curves, which were linear within a range from 0 to 50 μg ml^{-1} for both 4-*n*-NP and NP.

2.4. Sample preparation for light microscopy

Fungal pellets were obtained for analyses at 24, 48 and 72 h incubation and stained with Lactophenol blue solution (Fluka, Switzerland) according to the manufacturer's protocol. Images were captured using an AxioCam HR camera (Carl Zeiss, Germany) mounted on an inverted microscope, Axiovert 200M (Carl Zeiss, Germany), equipped with an Axioplan 20× objective lens.

2.5. Morphological measurements

The fungal pellet morphology was characterized using image analysis according to Cox and Thomas (1992). At least 50 pellets from each incubation point were used in the analysis. Image analyses were performed using the software package Axiovision 4.4 (Carl Zeiss, Germany). During the analyses a central compact core region and a peripheral "hairy" region of *M. robertsii* pellets were separated as previously described (Cox and Thomas, 1992; Casas Lopez et al., 2005). Because most *M. robertsii* pellets had a non-spherical shape, the obtained values of the pellet and pellet core projected area (mm^2) were estimated.

2.6. Sample preparation for transmission electron microscopy

For electron-microscopic analyses, the material was fixed using 3% glutaraldehyde in 0.2 M cacodylate buffer, pH = 6.0 for 2 h at 4 °C, then rinsed using the same buffer and postfixed in 1% osmium tetroxide for 2 h. After dehydration in a graded ethanol series, then in propylene oxide, the samples were gradually infiltrated and finally embedded in an Epon–Spurr's resin mixture. Ultrathin sections, prepared using a Reichert–Jung ultramicrotome, were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a JEOL JEM 1010 transmission electron microscope (TEM) at 80 kV.

2.7. Viability measurements

Fungal culture viability was assessed using a modified FDA (fluorescein diacetate) method (Hassan et al., 2002; Lecault et al., 2007). Briefly, whole *M. robertsii* cultures were obtained for analysis at 24 h incubation periods. Each sample was centrifuged at 1000×*g* for 10 min. After supernatant removal, the fungal cultures were suspended in 0.1 M phosphate buffer (pH = 4.5). Subsequently, each sample was placed in a separate well in a 24-well plate (Nunc, USA). A solution of 0.7 mg ml^{-1} FDA in acetone was prepared according to Lecault et al. (2007). Prior to the analysis, FDA stock solutions were diluted 20-fold in 0.1 M phosphate buffer (pH = 4.5). Three volumes of FDA working solution were added to each well. The plates were placed in the dark at 28 °C for 15 min. After incubation the fluorescence was read at an excitation of 485 nm and emission of 535 nm in a fluorescence microplate reader (Victor2 multifunctional counter; Wallac, Finland). After the measurements, fungal samples were obtained for dry mass analysis as previously described (Różalska et al., 2010). The data obtained from the microplate reader (mean fluorescence units) were divided by the dry mass amount obtained for each sample. The results were expressed as % viability, where the controls for each incubation time were set at 100%.