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Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions

Justyna Nykiel-Szymańska, Przemysław Bernat, Mirosława Słaba*

Department of Industrial Microbiology and Biotechnology, Faculty of Biology and Environmental Protection, University of Lodz, Banacha Street 12/16, Lodz 90-237, Poland

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ABSTRACT

The filamentous fungus *Trichoderma koningii* is capable of fast and effective eliminate alachlor (90% after 72 h when added separately and 80–60% in the presence of 1–5 mM of copper). After 168 h over 99% elimination of alachlor resulted in detoxification and was connected with the mitigation of reactive oxygen species (ROS) production. Using MS/MS techniques, seven dechlorinated and hydroxylated metabolites were identified. Cytochrome P450 and laccase participate in biotransformation of the herbicide by this non-ligninolytic fungus. Laccase activity is stimulated both by copper and the mixture of copper and alachlor, which seems to be important for combined pollutants. *T. koningii* is characterized by high tolerance to copper (up to 7.5 mM). The metal content in mycelia reached 0.9–7.76 mg in 1 g of dry biomass. Our results suggest that *T. koningii* strain seems to be a promising tool for bioremediation of agricultural areas co-contaminated with copper-based fungicides and chloroacetanilide herbicides.

1. Introduction

Alachlor [2-chloro-*N*-2,6-diethylphenyl-*N*-(methomethyl)acetamide] is a chloroacetanilide herbicide registered for pre- and post-emergent control of annual grasses and broadleaf weeds of corn, soybeans, cotton, sorghum, peanuts, rapeseed, or beans. Its huge consumption has caused negative impacts on both agricultural ecosystems and aquatic environment (Rattanawong et al., 2015). Alachlor toxicity is primarily due to its good solubility in water (240 mg L^{-1}), which contributes to its migration in the environment. Penetration of this compound into surface water and groundwater poses a threat to living organisms. Alachlor has been classified by Environmental Protection Agency (EPA, US) as a potential carcinogen of B2 group (EPA 1998). Furthermore, due to its toxic and genotoxic properties, the herbicide has been included in the group of compounds disrupting proper functioning of the endocrine system of humans and animals (EDC's) (UNEP and WHO, 2012). Therefore, there is a great concern regarding the fate of this herbicide and its byproducts in the environment. Alachlor can be degraded through physicochemical methods, but microbial degradation has a crucial relevance in its transformation in the natural environment. In the polluted areas, degradation is performed by a selected group of microflora that are capable of surviving in the presence of toxic substances (Wang et al., 2008; Xu et al., 2008). Till date, various microorganisms that are capable of degrading alachlor under both aerobic and anaerobic conditions have been isolated and identified. A lot of attention has been paid to bacteria originating from acetanilide-contaminated sludge and soil. Most of them belong to *Agrobacterium* sp., *Ancylobacter* sp., *Burkholderia* sp. (Ewida, 2014), *Bacillus* sp. (Wang et al., 2008; Xu et al., 2008), *Microbacterium* sp., *Pseudomonas* sp. (Xu et al., 2008) and *Streptomyces* sp. (Sette et al., 2004). However, only a limited number of studies have involved selected filamentous fungi that can effectively remove alachlor, for example, *Phanerochaete chrysosporium* (Ferrey et al., 1994; Chirnside et al., 2011), *Cunninghamella elegans* (Pothuluri et al., 1993), *Chaetomium globosum* (Tiedje and Hagedorn, 1975), *Paecilomyces marquandii* (Słaba et al., 2013c), *Candida xestobii* (Munoz et al., 2011), and *Coriolus versicolor* (Hai et al., 2012).

Xenobiotics degradation by soil microflora can be limited by the presence of excessive concentrations of heavy metal ions. Copper is one of the heavy metals having an adverse effect on the microbial processes such as cellular polymer synthesis, respiration, and cell division. In addition, it disturbs enzyme activity, membrane permeability, and can induce oxidative stress (Iwinski et al., 2017; Słaba et al., 2013a, 2013b). Copper compounds are often used as effective fungicides to protect infected plants as they are highly effective against fungi (Filimonova et al., 2018). Since 1904, copper has also been used as an aquatic herbicide and algaecide (Iwinski et al., 2017). In contrast, it is a beneficial micronutrient needed to achieve the appropriate height and

* Corresponding author.
E-mail address: miroslawa.slaba@biol.uni.lodz.pl (M. Słaba).

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quality in the case of many crops (Zou et al., 2015).

Nonspecific oxidative enzymes, for example, laccase, lignin peroxidase, manganese peroxidase, and cytochrome P450 oxidase are often engaged in the fungal elimination of many xenobiotics e.g., polycyclic aromatic hydrocarbons (PAHs) (Bautista et al., 2015), chlorophenols, pesticides (Rao et al., 2014) and dyes (Jasińska et al., 2015). Laccase activity in non-ligninolytic fungi from the genus *Trichoderma* has been described by Chakroun et al. (2010); Sadhasivam et al. (2008), and Wang et al. (2012). Fungal laccases have been used in many processes, for example, decolorization or synthesis of dye (Jasińska et al., 2015; Polak and Jarosz-Wilkolazka, 2012), biopulping, delignification of ligninocellulosis compounds, production of ethanol, treatment of wastewater (Chen et al., 2015b), and degradation of pesticides (Khambhaty et al., 2015). Elimination of alachlor with the participation of the enzyme laccase was observed in the lignin-degrading white rot fungus *P. chrysosporium* (Chirnside et al., 2011), whereas degradation of chloroacetanilides by non-ligninolitic fungi with laccase is so far undocumented. The role of cytochrome P450 (CYP450) in alachlor metabolism was detected in different eukaryotic organisms such as fungi (*C. elegans*) (Pothuluri et al., 1993) and mammalians (rats) (Kale et al., 2008).

Filamentous fungi from the genus *Trichoderma* are cosmopolitan microorganisms, mostly isolated from the soil or plant roots These fungi are best known for supporting the growth of plants and protecting them from pathogens (Tripathi et al., 2013). Their prevalence in the environment is primarily due to their ability to grow rapidly, to propagate in adverse environmental conditions, as well as their ability to use different sources of carbon and nitrogen (Raspanti et al., 2009). Because of their high metabolic activity, the fungi are able to degrade a broad spectrum of xenobiotics, for example PAHs, cyanides, synthetic dyes, cresols, phenols, and plant protection products (e.g., dicofol, dichlorodiphenyltrichloroethane (DDT), dichlorvos, and chlorpyrifos) (Tripathi et al., 2013).

In this study, we investigated the transformation and detoxification of alachlor by *Trichoderma koningii* with the involvement of laccase and the cytochrome P450 system. Moreover, due to the high tolerance of *T. koningii* to copper ions, its ability to simultaneously eliminate alachlor and copper ions was determined.

2. Materials and methods

2.1. Chemicals

Alachlor (PESTANAL®, analytical standard (99.2%)), metabolic inhibitors, laccase substrate – 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), copper sulfate, nitrotetrazolium blue chloride (NBT) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (Poznan, Poland). Ethyl acetate, for alachlor extraction, was purchased from POCh (Gliwice, Poland), and high purity solvents used during sample preparation for high pressure liquid chromatography (HPLC) were obtained from Sigma-Aldrich (Poznan, Poland) and POCh (Gliwice, Poland).

2.2. Microorganism and growth conditions

The filamentous fungus *Trichoderma koningii* IM 0956 was obtained from the Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, Poland. Seven-day-old spores of *T. koningii* strain from cultures on ZT agar plants (g L^{-1}: glucose (4); Difco yeast extract (4); agar (25); and malt extract (6 °Blg), up to 1 L; pH 7.0) were used to inoculate 20 mL of mineral medium with 2% glucose and 1% yeast extract in 100-mL Erlenmeyer flasks. The medium was composed of (g L^{-1}): K_2HPO_4 (4.36), KH_2PO_4 (1.7), NH_4Cl (2.1), $MgSO_4 \times 7H_2O$ (0.2), $MnSO_4$ (0.05), $FeSO_4 \times 7H_20$ (0.01), $CaCl_2 \times 2H_2O$ (0.03), glucose (20), yeast extract (10), and distilled water (up to 1 L, pH 6.5). The fungal cultures were grown on a rotary shaker (160 rpm) for 24 h at 28 °C. The preculture was transferred to a fresh medium in the ratio of 1:1 and incubated for the next 24 h. The homogenous preculture (10%) was introduced into the medium supplemented with 50 mg L^{-1} alachlor dissolved in ethanol (stock solution 10 mg mL^{-1}) or a control culture without the tested compound. In addition, abiotic controls (uninoculated) were also prepared. All cultures were incubated at 28 °C on a rotary shaker (160 rpm). At appropriate time, culture samples were selected for analyses.

2.3. Determination of alachlor and its metabolites

Samples were prepared according to the method described by Słaba et al. (2013c) with some modifications. The cultures were homogenized with glass beads twice for 4 min at 25 m s^{-1} (Retsch, Ball Mill MM 400). Next, the samples were extracted twice with ethyl acetate. The extracts were dried with anhydrous sodium sulfate and evaporated under reduced pressure at 40 °C. Evaporated residues were dissolved in 2 mL of methanol and 0.2 mL was transferred to chromatography plates for quantitative and qualitative analyses.

Quantitative analysis of alachlor was performed on the Agilent 1200 LC System coupled with an AB Sciex 3200 QTRAP mass detector equipped with a TurboSpray Ion Source (electrospray ionization (ESI)). The separation was performed in a capillary Eclipse XDB-C18 column (50 mm × 4.6 mm, 4.6 μm) maintained at 37 °C. The mobile phase consisted of water (A) and methanol (B) supplemented with 5 mmol L^{-1} ammonium formate. The run time was 6 min with the solvent gradient initiated at 50% B. After 1 min, during the next minute, B was decreased to 5% and maintained at 5% for four additional minutes before returning to the initial solvent composition over 2 min. The flow rate was 600 μL min^{-1}. The detection of alachlor was performed on an MS/MS detector working in the multiple reaction monitoring (MRM) positive ionization mode. The monitored MRM pairs were *m/z* 270.1–238.2 and *m/z* 270.1–162.3 at 1.61 retention time. The quantitative analysis of alachlor in the tested samples was performed according to the standard equation ($y = 6.67e+0.006\times$; $r = 0.991$), and the alachlor content was calculated in percentage. A 100% value represented the amounts of alachlor, which were estimated in abiotic controls.

Samples for qualitative analysis were selected from 0, 24, 72, 120, and 168 h of culture and included corresponding biotic and abiotic controls acting as a reference for the detection of alachlor metabolites. The same chromatographic method was used to identify the possible alachlor metabolites, which were detected in previously extracted samples. The mass spectrometric analysis was performed using a targeted scanning mode. According the previous data on alachlor biodegradation (Słaba et al., 2013c), a list of predicted Multiple Reaction Monitoring (pMRM) pairs of possible metabolites in positive ionization mode was created. The pMRM-based method was constructed based on the characteristic fragmentations occurring in the alachlor mass spectrum: *m/z* 238, *m/z* 224, *m/z* 220, *m/z* 210, *m/z* 208, *m/z* 90, and *m/z* 77. Then, Information Dependent Acquisition (IDA) criteria were employed to automatically trigger the acquisition of full-scan MS/MS spectra for any compounds that were detected by the pMRM scans. The Enhanced Product Ion (EPI) scan-type was used to acquire full-scan MS/MS spectra in the range of *m/z* 50–350 in the linear ion trap of the QTRAP system. The optimized ion source parameters were: CUR: 25, IS: 5500 V, TEMP: 550 °C, GS1: 55 psi, GS2: 50 psi.

2.4. Toxicity study

The possible toxicity of postculture extracts of the *T. koningii* strain, cultivated with or without alachlor was examined using a commercial bioassay Artoxkit M provided by MicroBioTests, Inc., Belgium. Experiments were based on the immobilization of the larvae of *Artemia franciscana* in accordance with standard operational procedures. The