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# Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi – Metabolites, enzymes and residual antibacterial activity

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## HIGHLIGHTS

- Fluoroquinolone antibiotics are easily degradable by ligninolytic fungi.
- *Irpex lacteus* and *Trametes versicolor* degraded fluoroquinolones most efficiently.
- The compounds are attacked at the piperazinyl moiety: substitution or decomposition.
- Only *I. lacteus* removed the antibiotic activity during the degradation.
- PCA indicated that manganese peroxidase might participate in the degradation.

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## ABSTRACT

A group of white rot fungi (*Irpex lacteus*, *Panus tigrinus*, *Dichomitus squalens*, *Trametes versicolor* and *Pleurotus ostreatus*) was investigated for the biodegradation of norfloxacin (NOR), ofloxacin (OF) and ciprofloxacin (CIP). The selected fluoroquinolones were readily degraded almost completely by *I. lacteus* and *T. versicolor* within 10 and 14 d of incubation in liquid medium, respectively. The biodegradation products were identified by liquid chromatography–mass spectrometry. The analyses indicated that the fungi use similar mechanisms to degrade structurally related antibiotics. The piperazine ring of the molecules is preferably attacked *via* either substitution or/and decomposition. In addition to the degradation efficiency, attention was devoted to the residual antibiotic activities estimated using Gram-positive and Gram-negative bacteria. Only *I. lacteus* was able to remove the antibiotic activity during the course of the degradation of NOR and OF. The product-effect correlations evaluated by Principal Component Analysis (PCA) enabled elucidation of the participation of the individual metabolites in the residual antibacterial activity. Most of the metabolites correlated with the antibacterial activity, explaining the rather high residual activity remaining after the biodegradation. PCA of ligninolytic enzyme activities indicated that manganese peroxidase might participate in the degradation.



## 1. Introduction

Fluoroquinolones represent a group of xenobiotics that are often monitored in the environment. These compounds are important antibacterial agents used in human and veterinary medicine; however, their presence in the environment can lead to the selection of resistant pathogenic bacterial strains (Hayes et al., 2004). These antibiotics enter the environment mainly via wastewater (Nakata et al., 2005; Mitani and Kataoka, 2006; Lee et al., 2007), have been found in surface waters (Speltini et al., 2010) and can also be accumulated in sewage sludge (Lillenberg et al., 2009; Li and Zhang, 2010; Jia et al., 2012), sediments or agricultural soils after application of sewage sludge or manure (Speltini et al., 2011). Sequestration of fluoroquinolones by sorption onto soil particles reduces their bioavailability, retards their abiotic and biotic degradation and therefore increases their persistence in soils and aquatic sediments (Sukul and Spiteller, 2007; Girardi et al., 2011). However, sorption on soil can also significantly decrease the toxicity of pollutants, as was demonstrated for polycyclic aromatic hydrocarbons (Čvančarová et al., 2013).

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Photodegradation is the main abiotic process that eliminates antibiotics in aquatic systems such as surface waters (Cardoza et al., 2005; Sturini et al., 2012). Despite the fact that antibiotics are antimicrobial agents, microbial biotransformation represents another important mechanism in the elimination of these drugs from the environment. Microbial transformation of quinolones was reviewed by Parshikov and Sutherland (2012). Norfloxacin was found to be transformed by *Mycobacterium gilvum* to *N*-acetylnorfloxacin and *N*-nitrosonorfloxacin. However; although *N*-nitrosonorfloxacin has lower antibacterial activity, nitrosamines are potentially carcinogenic (Adjei et al., 2006). Another degradation mechanism of norfloxacin was described by Kim et al. (2011) who carried out the biodegradation using the *Microbacterium* sp. strain isolated from a wastewater treatment plant. Regarding fungal degradation, the biotransformation of selected fluoroquinolones mainly by a brown rot fungus was described by Wetzstein et al. (1999). Flumequine degradation was studied using the zygomycete *Cunninghamella elegans* (Williams et al., 2007) and transformation of ciprofloxacin, enrofloxacin and sarafloxacin by the saprobic fungus *Mucor ramannianus* was investigated by Parshikov et al. (1999, 2000, 2001b). The ability of the nonpathogenic fungus *Pestalotiopsis guepini* to metabolize norfloxacin was also studied (Parshikov et al., 2001a,b; Williams et al., 2004). Another promising group of fungi that are able to transform recalcitrant compounds and possess a unique set of extracellular ligninolytic enzymes are ligninolytic fungi (Muzikář et al., 2011; Čvančarová et al., 2012; Křesinová et al., 2012). However, little is known about the biotransformation of fluoroquinolones by white rot fungi. Marengo et al. (1997) investigated the biodegradation of $^{14}$C-labeled sarafloxacin hydrochloride by the fungus *Phanerochaete chrysosporium* and Prieto et al. (2011) metabolized norfloxacin and ciprofloxacin by *Trametes versicolor*, where the latter authors identified several transformation products of the antibiotics. In a recent study, a transformation pathway of flumequine by several white rot fungi was suggested (Čvančarová et al., 2013).

The purpose of this study was first to elaborate knowledge of the degradation potential of white rot fungi for fluoroquinolones and secondly to assess possible environmental risks following from the biodegradation processes. The fungal strains *Irpex lacteus*, *Panus tigrinus*, *Dichomitus squalens*, *T. versicolor* and *Pleurotus ostreatus* were selected to biodegrade norfloxacin (NOR), ofloxacin (OF) and ciprofloxacin (CIP) in liquid medium. The bacteria *Vibrio fischeri* and duckweed *Lemna minor* were used as test assays to consider possible changes in ecotoxical effects related to the biodegradation. In addition, the residual antibiotic activity of the biodegradation product mixtures was estimated using several Gram-positive and Gram-negative bacteria. Multivariate data analysis was performed to elucidate correlations between identified metabolites and the remaining antibacterial activity measured in the sample solutions. An attempt was also made to estimate possible participation of ligninolytic enzymes in the degradation.

## 2. Materials and methods

### 2.1. Chemicals

The analytical standards of the individual antibiotics (norfloxacin – NOR, CAS No 70458-96-7, ⩾98%; ofloxacin – OF, CAS No 82419-36-1, ⩾99%; ciprofloxacin – CIP, CAS No 85721-33-1, ⩾98%) were obtained from Sigma Aldrich (Steinheim, Germany). All the solvents were purchased from Merck (Darmstadt, Germany) or Chromservis (Prague, Czech Republic) and were of p.a. quality, trace analysis quality or gradient grade.

### 2.2. Biodegradation experiment, microorganisms and measurement of enzyme activities

The selected antibiotics were degraded separately by the white rot fungi *I. lacteus* 617/93, *P. tigrinus* 577.79, *D. squalens* CCBAS 750, *T. versicolor* 167/93 and *P. ostreatus* 3004 CCBAS 278 obtained from the Culture Collection of Basidiomycetes of the Academy of Science, Prague. The fungi were cultivated in malt extract–glucose medium (MEG) according to Cajthaml et al. (2006). Briefly, the cultures in 250 mL Erlenmeyer flasks containing 20 mL of media were spiked with a solution of the respective antibiotic in 100 μL dimethyl sulfoxide reaching a final concentration of 10 μg mL$^{-1}$. The cultures were cultivated in the dark at 28 °C in five replicates under static conditions. The samples were analyzed using HPLC after 3, 6, 10 and 14 d, while 100 μL of the medium were diluted with methanol and injected directly into the HPLC. The heat-killed controls were performed following one week of growth of the fungal culture (*I. lacteus*), which was killed in an autoclave (120 °C, 1.5 h) before addition of the respective target substance. The abiotic controls were prepared by addition of the antibiotics to the MEG medium without mycelium. The abiotic controls were cultivated under the same conditions to exclude chemical or photodegradation of the antibiotics in the medium.

The ligninolytic enzyme activities of lignin peroxidase (LiP), manganese peroxidase (MnP), manganese-independent peroxidase (MIP) and laccase (Lac) were determined as described previously. LiP (E.C. 1.11.1.14) was assayed with veratryl alcohol as the substrate (Dejong et al., 1994) and MnP (E.C. 1.11.1.13) was determined with 2,6-dimethoxyphenol (Matsumura et al., 1986). Lac (E.C. 1.10.3.2) was estimated with 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid as the substrate (Covino et al., 2010). MiP was calculated from the peroxidase activity of the MnP assay detected in the absence of $Mn^{2+}$ ions (Cajthaml et al., 2008).

### 2.3. Quantitative analysis by HPLC

The residual concentrations of the antibiotics in the liquid media during and after the treatment were determined by RP-HPLC consisting of a 2695 Separations Module (Waters, Prague, Czech Republic), a 2996 diode-array detector and a column X-Bridge C18 (250 mm × 4.6 mm; particle size 3.5 μm; waters). Separation of the antibiotics was achieved with gradient elution using (A) 0.1% trifluoroacetic acid in 10% acetonitrile and (B) 100% methanol. The elution program started with 20% (B) and was followed by a linear gradient to 55% (B) in 11 min. The separation temperature was set at 25 °C and the flow rate at 0.7 mL min$^{-1}$. The compounds were quantified on the basis of the UV spectra measured in the range 220–400 nm ($NOR_{max}$ 279 nm, $OF_{max}$ 294 nm, $CIP_{max}$ 278 nm). Calibration curves were constructed in the concentration range 0.1–50 μg mL$^{-1}$ for each compound.

### 2.4. Identification of degradation products

The biodegradation products were identified by HPLC-MS/MS after 14 d of incubation. 20 μL of the liquid growth medium were injected automatically into the HPLC (Agilent 1100 series, Agilent Technology, Santa Clara, CA, USA). The separation was performed on a TSKgel ODS-100V column (150 mm × 4.6 mm; particle size 5 μm; Tohos Bioscience, Stuttgart, Germany) at a temperature of 25 °C and the flow rate was set at 0.2 mL min$^{-1}$. The mobile phase consisted of (A) 0.1% formic acid, 5% acetonitrile, 95% bidistilled water and (B) 0.1% formic acid, 95% acetonitrile, 5% bidistilled water. The elution program comprised isocratic elution with 10% (B) for 1 min followed by a gradient to 100% (B) in 30 min. The mass spectrometer (API 2000, AB Sciex, Darmstadt, Germany) coupled to the HPLC was equipped with a triple quadrupole and