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Continuous degradation of a mixture of sulfonamides by *Trametes versicolor* and identification of metabolites from sulfapyridine and sulfathiazole

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ABSTRACT

In this study, we assessed the degradation of the sulfonamides sulfapyridine (SPY) and sulfathiazole (STZ) by the white-rot fungus *Trametes versicolor*. Complete degradation was accomplished in fungal cultures at initial pollutant concentrations of approximately 10 mg L^{-1}, although a longer period of time was needed to completely remove STZ in comparison to SPY. When cytochrome P450 inhibitors were added to the fungal cultures, STZ degradation was partially suppressed, while no additional effect was observed for SPY. Experiments with purified laccase and laccase mediators caused the removal of greater than 75% of each antibiotic. Ultra-performance liquid chromatography-quadupole time of flight mass spectrometry (UPLC-QqTOF-MS) analyses allowed the identification of a total of eight degradation intermediates of SPY in both the *in vivo* and the laccase experiments, being its desulfonated moiety the commonly detected product. For STZ, a total of five products were identified. A fluidized bed reactor with *T. versicolor* pellets degraded a mixture of sulfonamides (SPY, STZ and sulfamethazine, SMZ) by greater than 94% each at a hydraulic residence time of 72 h. Because wastewater contains many diverse pollutants, these results highlight the potential of *T. versicolor* as a bioremediation agent not only for the removal of antibiotics but also for the elimination of a wide range of contaminants.

1. Introduction

Because of their low cost and relative efficiency in combating many common bacterial infections, sulfonamides (SAs) are some of the most widely used antibiotics [1]. In the EU, sulfonamides are the second most widely used class of veterinary antibiotics after tetracyclines [2]. SAs are sometimes used to treat human diseases, but they are more commonly used in veterinary medicine, especially on animal and fish farms. However, the spread of microbial resistance has raised concerns about the prevalence of SAs in the environment [3,4]. The excretion of SAs in the feces and urine of medicated animals and the subsequent application of the contaminated manure as fertilizer onto agricultural land are among the major routes through which SAs enter the environment. Previous studies showed that livestock excrete up to 50–90% of the administered dose, the parent drug making up ~9–50% of the excreted products [5,6]. SAs are highly soluble and weakly acidic, which allows them to be leached from the soil and run off into ground and surface waters [7,8]. Other environmental sources of SAs include aquaculture, hospital effluents, and the disposal of unused drugs from WWTPs, where SAs elimination is often incomplete [9,10].

Alternative eco-friendly treatments to remove organic pollutants such as SAs are of great interest. The white rot fungus (WRF) *Trametes versicolor* has the potential to remove a diverse range of xenobiotics [11], even from complex matrices such as sludge [12], which due to its extracellular and non-specific lignin-mineralizing enzymes (*i.e.*, laccases and peroxidases) and intracellular enzymatic complexes (*e.g.*, cytochrome P450) [13]. Previous studies have demonstrated the laccase-mediated processes of transformation of SAs in liquid medium and their coupling to organic matter [14–16]. However, bioreactor-scale approaches for the application of WRF in the bioremediation of emerging pollutants are still scarce [17].

This work aimed at demonstrating the ability of *T. versicolor* to degrade two SAs, sulfapyridine (SPY) and sulfathiazole (STZ),

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through the identification of their metabolites and the determination of the role of specific enzymatic systems in the transformation process. Once described the degradation, the feasibility of the simultaneous elimination of a mixture of three SAs (SPY, STZ and sulfamethazine, SMZ) was also evaluated in a continuous fluidized bed reactor with fungal pellets.

2. Materials and methods

2.1. Fungal strain

The strain *T. versicolor* (ATCC 42530) was acquired from the American Type Culture Collection and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 25 °C. *T. versicolor* pellets were produced as described by Font Segura et al. [18].

2.2. Chemicals and reagents

SPY (4-amino-*N*-(2-pyridinyl)benzenesulfonamide, 99%), STZ (4-amino-*N*-(2-thiazolyl)benzenesulfonamide, Vetranal®, 99.9%), SMZ (4-amino-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide, 99%), piperonyl butoxide (PB, ≥90%), 1-aminobenzotriazole (ABT), 3,5-dimethoxy-4-hydroxyacetophenon (DMHAP, 97%), 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, ∼98%), violuric acid (VA, ≥97%) and purified laccase from *T. versicolor* were obtained from Sigma-Aldrich (St. Louis, MO, USA). The internal standard d_4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada). High performance liquid chromatography (HPLC)-grade acetonitrile, water and formic acid (98%) were purchased from Merck (Darmstadt, Germany). Nylon filters (0.45 μm) were purchased from Whatman (Maidstone, UK). The Hydromatrix dispersing agent was purchased from Agilent (Santa Clara, CA, USA).

2.3. Experimental procedures

2.3.1. In vivo degradation experiments

The degradation experiments were performed in 250 mL Erlenmeyer flasks containing 10 g of fungal pellets (wet weight) in a total volume of 50 mL of a chemically defined medium at pH 4.5 (composition per liter: 8 g glucose, 498 mg nitrogen as ammonium tartrate, 10 and 100 mL micro- and macronutrient solutions [19], respectively, and 1.168 g 2,2-dimethylsuccinate). Uninoculated flasks containing 50 mL of defined medium and autoclaved cultures were employed as abiotic and heat-killed controls, respectively. All of the conditions were tested in triplicate. SPY or STZ was added from a stock solution in methanol to give the desired concentration (approximately 9–11 mg L^{-1}). The flasks were incubated in the dark on an orbital shaker (135 rpm) at 25 °C. In the time course experiments, 1 mL samples were periodically withdrawn, filtered (0.22 μm Millex-GV filters, Millipore, Billerica, MA) and subsequently analyzed by HPLC. Adsorption was estimated by comparing the concentration of the SAs in the heat-killed controls with the concentration in the abiotic controls. Degradation percentages were determined using the concentration values in the heat-killed controls as a baseline.

2.3.2. Experiments with cytochrome P450 inhibitors and enzymatic degradation with laccase

To determine the effect of cytochrome P450 inhibitors, PB or ABT was added to a final concentration of 5 mM in the experiments performed as described in Section 2.3.1. The assays were performed in triplicate. Laccase-mediated degradation experiments were performed in Erlenmeyer flasks containing 50 mL of a purified laccase solution (pH 4.5) at an initial activity concentration of 50.4 ± 8.2 activity units (U) L^{-1} for SPY and 55.4 ± 9.3 U L^{-1} for STZ. The effect of laccase mediators was evaluated by adding VA, DMHAP or ABTS (0.8 mM each) to the reaction mixture. Controls containing milli-Q water at pH 4.5 were included in the analysis. SPY was added at a concentration of 20 mg L^{-1}, and STZ was added at a concentration of 16 mg L^{-1}. The flasks were incubated in the dark on an orbital shaker (135 rpm) at 25 °C. At designated time points, 1 mL samples were withdrawn, and 100 μL of acetic acid was added to each flask to stop the reactions prior HPLC analysis.

2.3.3. Continuous degradation of SAs in a fluidized bed reactor

A fungal pellet fluidized bed bioreactor [20] was employed to degrade a mixture of SAs. The working volume was set at 1500 mL. Fluidized conditions were maintained by air pulses generated by an electrovalve that was alternately open for 1 s and a shut for 4 s. A pH controller was used to maintain the pH at 4.5 ± 0.2, and the system was kept at 25 °C. The bioreactor was initially loaded with 1.5 L of defined medium as described in Section 2.3.1, glucose (10 g L^{-1}) and SAs (SMZ, SPY and STZ, 5 mg L^{-1} each). The reactor was inoculated with fungal pellets at 2.3 g L^{-1} (dry cell weight, DCW) and operated in batch mode. Once the glucose concentration reached 1 g L^{-1}, the continuous stage was switched on with an initial hydraulic residence time (HRT) of 48 h; the HRT was changed according to the system's performance during the experiment. The feeding solution consisted of defined medium without glucose and SAs at 5 mg L^{-1} each. Glucose was supplied separately at the consumption rate (approximately 2 g L^{-1} d^{-1}, [20]). Throughout the experiment, the biomass was contained inside the reactor with a metal mesh in the outlet.

2.4. Analytical procedures

2.4.1. Analyses of SAs

SAs were quantified using a Dionex 3000 Ultimate HPLC (Sunnyvale, CA) equipped with a UV detector at 264 nm. Chromatographic separation was achieved at 30 °C by injecting 20 μL samples on a Grace Smart RP18 column (250 mm × 4 mm, 5 μm particle size); the mobile phase consisted of 40 mM ammonium acetate buffer (A, pH 7) and methanol (B). For the analysis of individual SAs, the eluents were added isocratically (65% A, 35% B) at 1 mL min^{-1} [21]. The retention times were 4.3 min and 3.9 min for SPY and STZ, respectively. The elution of the mixture of SAs in the reactor experiments was accomplished with a linear increase from 0% B to 35% B over 10 min, isocratic elution for 2 min, and then a return to the initial conditions in 2 min. The retention times in this case were 9.9 min (STZ), 10.6 min (SPY) and 12.1 min (SMZ).

2.4.2. Identification of degradation products

MS and tandem MS (MS/MS) analyses of SPY and STZ and its degradation products were performed using a Waters/Micromass QqTOF-Micro system coupled to a waters acquity ultra performance liquid chromatography (UPLC) system (Micromass, Manchester, UK). A Waters Acquity BEH C18 column (10 mm × 2.1 mm, 1.7 μm particle size) was employed. The flow rate was set up at 0.3 mL min^{-1}. Eluent A (HPLC-grade water) and eluent B (acetonitrile) were both acidified with 10 mM of formic acid. The elution was performed with a linear increase of B from 5% to 60% over 7 min, a further increase of B to 95% over the following 2 min, and then a return to the initial conditions in 2 min. The injection volume of the sample was 5 μL. The analyses were performed in the positive ionization (PI) mode. The operating conditions are described in a previous publication by the authors of this work [22]. For continuous internal mass calibration, an independent reference (valine–tyrosine–valine) was used as a lock mass, with m/z 380.2185, and was acquired in all of the measurements. For the MS