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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

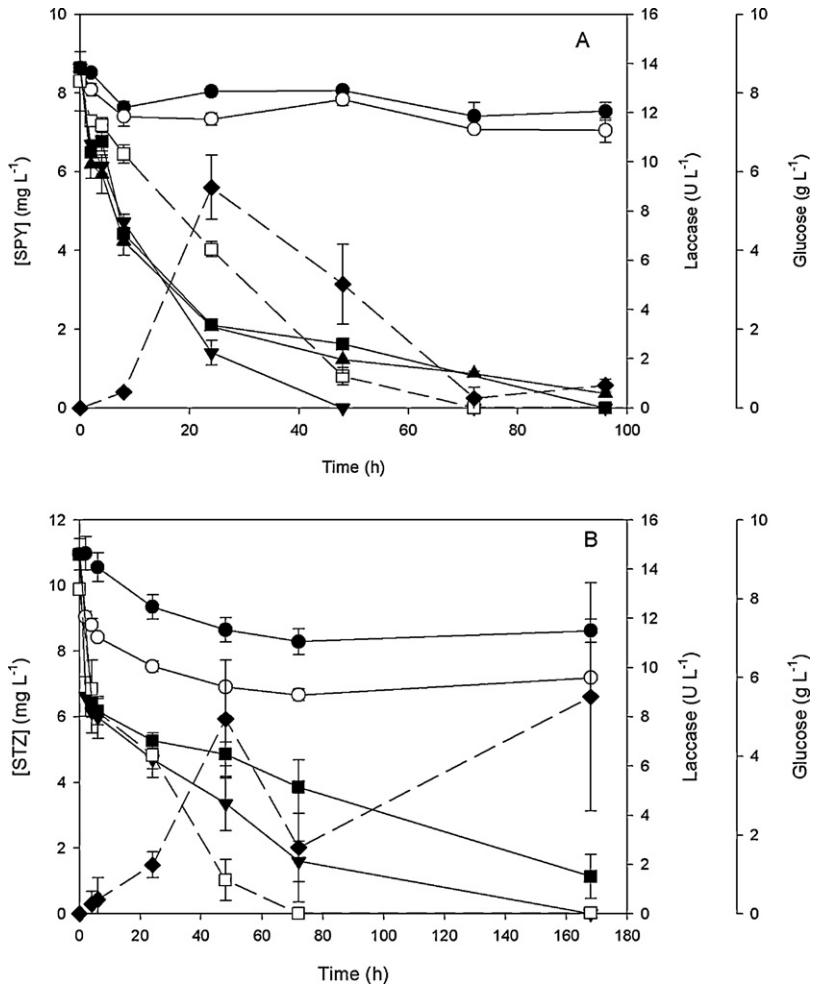

Fig. 1. Degradation profiles of SPY (A) and STZ (B) by *T. versicolor* at the bench scale. Symbols: uninoculated controls (●), heat-killed controls (○), reaction cultures (▼), cultures containing cytochrome P450 inhibitors PB (■) and ABT (▲), glucose consumption (□) and laccase activity (♦). Values plotted are the means ± standard deviation (SD) for triplicate cultures. The initial pellet biomass (dry weight) was 282.7 ± 5.2 mg and 353.7 ± 10.8 mg for SPY and STZ, respectively.

analyses on the QqTOF instrument, the MS data were collected by scanning from *m*/*z* 50 to *m*/*z* 500.

2.4.3. Other analyses

Laccase activity was measured as described by Wariishi et al. [23]; 2,6-dimethoxyphenol (DMP) was used as the substrate. The results are expressed as U L^{-1}. One activity unit is defined as the number of micromoles of DMP oxidized per min. The glucose levels were analyzed by the glucose oxidase method in a YSI 2700 analyzer (Yellow Springs, OH). The mycelia dry weight was determined by vacuum filtering the cultures through preweighed glass filters (Whatman GF/C). The filters containing the biomass were dried at 100 °C to constant weight.

3. Results and discussion

3.1. Degradation of SPY and STZ by T. versicolor

The fungal transformation of the SAs SPY and STZ was assayed and the degradation profiles are shown in Fig. 1. Complete removal of SPY was achieved after 48 h (Fig. 1A), although most of the degradation occurred during the first 24 h (81 ± 5%). The experimental degradation rate was 86.5 ± 5.5 ng h^{-1} mg^{-1} DCW (0.347 ± 0.022 nmol h^{-1} mg^{-1} DCW). Adsorption, which was determined by comparing the concentrations in the heat-killed controls and the uninoculated flasks, was negligible. STZ (Fig. 1B) removal was slightly slower, and more than 20% was still detected after 72 h, although the initial elimination rate was higher than with SPY (117.3 ± 19.2 ng h^{-1} mg^{-1} DCW, 0.459 ± 0.075 nmol h^{-1} mg^{-1} DCW). The adsorption to heat-killed biomass was 17 ± 4%. A slight reduction in the concentration of both SAs was observed in the uninoculated flasks, which can be ascribed to the production of N^4-glycosyl-SPY and N^4-glucosyl-STZ as by-products released due to the presence of glucose in the defined medium. These compounds were detected and confirmed by MS and tandem MS/MS analyses; an analogous phenomenon has been described for SMZ [22]. The confirmation of degradation metabolites in the reaction cultures, discussed in Section 3.3, indicates that the elimination of SPY and STZ, as demonstrated for the elimination of SMZ [22], is due to a degradation process and cannot be ascribed solely to sorption phenomena.

3.2. Effect of cytochrome P450 inhibitors and the role of laccase in the degradation of SAs

Cytochrome P450, an intracellular enzymatic complex, has been implicated in the degradation of several organic pollutants by WRF [24,25]. To evaluate its role in the transformation of the SAs, the cytochrome P450 inhibitors PB and ABT were employed [26]. When comparing the inhibitor-containing cultures and the reaction cultures in Fig. 1, no difference was observed in the first hours, possibly corresponding to the period of active transport of SAs into the

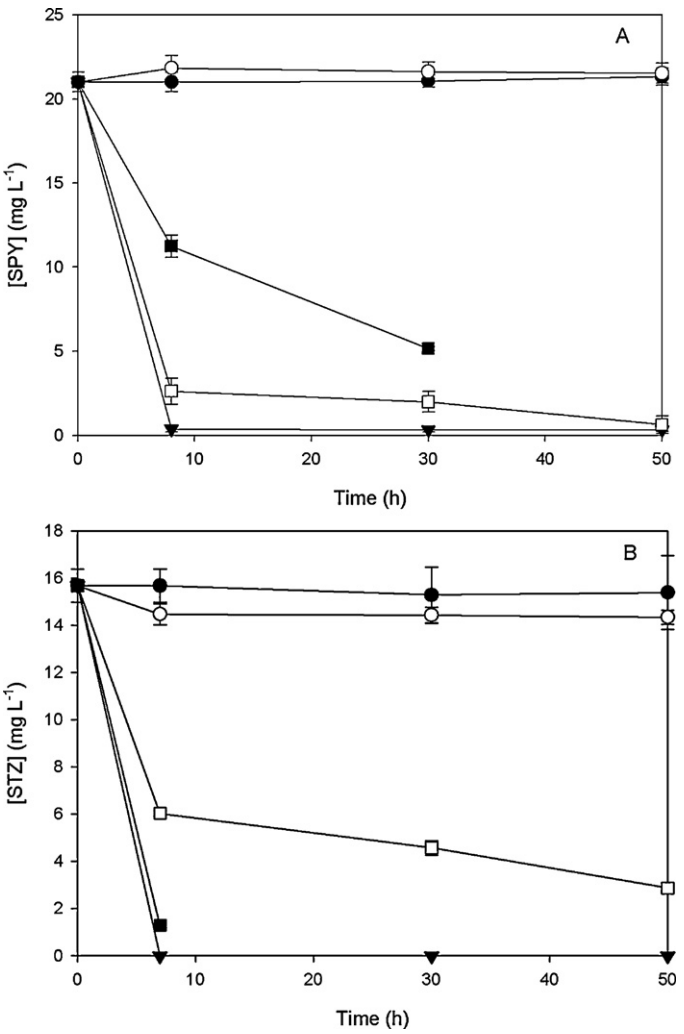

Fig. 2. Degradation profiles of SPY (A) and STZ (B) by purified laccase. Symbols: laccase-free controls (●), laccase without mediators (○), laccase with VA (▼), laccase with DMHAP (□) and laccase with ABTS (■). Values plotted are the means ± SD for triplicate cultures.

fungal cells. There was a slight delay in the degradation of SPY (24 h) in the inhibitor-containing cultures, but almost complete removal (>95%) was obtained after 96 h. On the other hand, a decelerated decline in STZ concentration was observed in the cultures with PB, if compared to inhibitor-free cultures. These results suggest that the cytochrome P450 complex may be involved in the removal of STZ, whereas there is no clear experimental evidence that cytochrome P450 plays a role in the degradation of SPY.

Similar to cytochrome P450, the participation of laccase in the removal of diverse groups of environmental contaminants has been widely investigated [27]. Because laccase activity levels up to 10 U L^{-1} were obtained in the degradation assays (Fig. 1), further experiments that focused on determining the role of this enzyme were performed with purified laccase and laccase mediator systems [13]. Enzymatic degradation with solely commercial laccase (Fig. 2) caused minimal SA degradation in 50 h. However, when VA, DMHAP and ABTS—mediators in laccase reactions—were applied, the elimination of the antibiotics ranged from 75% to 98% (±4%) for SPY and 82% to 100% (±3%) for STZ by the end of the experiment. Laccase mediators favor the oxidation of non-phenolic compounds [28], and although they are not produced by *T. versicolor* itself, molecules with analogous functions may be released during active growth. This, together with the combined activity of other enzymatic systems (*e.g.* cytochrome P450), results in shorter treatment periods with the use of whole fungal cells, compared to those reported by purified laccase, in which significant transformation of SAs has been achieved only with longer incubation times (9–16 d) [16]. Laccase-mediated degradation has been demonstrated for some pharmaceuticals, including naproxen and other SAs [16,22,29].

3.3. Identification of degradation products from sulfapyridine

3.3.1. In vivo experiments

SPY was fully degraded after 48 h in the fungal cultures, and the presence of 7 different transformation products confirmed this finding (Fig. 3). Although the relative mass errors of the degradation products were sufficiently accurate for identification, confirmation by MS/MS spectra was not always achieved, as sometimes no more than a single fragment could be obtained for each of them. In these cases, the predicted structures were confirmed with the double bond equivalent (DBE) values and the sodium adduct signals from the chromatograms (see Supplementary information, SI).

Upon chromatographic separation with the UPLC, SPY was detected at a retention time (RT) of 2.95 min. At reaction time 0, a peak at an RT of 2.20 min was already present in both the abiotic and heat-killed control samples and the reaction samples, with a base peak *m*/*z* of 412.1177 (average value, $n = 4$) in the corresponding mass spectra. The elemental composition for this mass was $C_{17}H_{22}N_3O_7S$ (SI, Table S1), corresponding to the by-product N^4-glycosyl-SPY (P6 in Fig. 3), which is formed due to the presence of glucose in the defined culture medium described in Section 2.3.1. To confirm this finding, a CE of 30 eV was applied, and stable fragment ions at *m*/*z* 255.1083 and *m*/*z* 108.0961 were obtained (SI, Fig. S1). In the reaction samples, this adduct was also present at a concentration profile similar to that of SPY, indicating that both were consumed by the fungi to a similar extent (Fig. 4A). The glycosylated adduct was subjected to transformation itself, and a second glycosylated product was detected after 8 h in the reaction samples, with a RT of 1.52 min and a base peak of *m*/*z* 348.1550 (mean value, $n = 3$) in the corresponding mass spectra. It was identified as the desulfonated product of N^4-glycosyl-SPY (P2).

A new peak appeared at a reaction time of 8 h and at a RT of 1.9 min, with an observed *m*/*z* of 186.1039 ($n = 4$). Its elemental composition was elucidated as $C_{11}H_{12}N_3$ and corresponded to the desulfonated product of SPY, reaching a maximum relative intensity of 80% after 24 h (Fig. 4, P4). The product ion spectrum (CE of 30 eV) yielded peaks at *m*/*z* of 108.0698 and 93.0574 (SI, Fig. S1). This desulfonated product of SPY was the only common metabolite present also in the enzymatic degradation experiments. Previous studies have demonstrated that desulfonated products are frequently detected after the enzymatic degradation, physicochemical oxidation or photodegradation of SAs [16,22,30–32].

Two different signals corresponded to the same mass and yielded the same mass spectra: P5 and P5′. Both stereoisomers appeared in the chromatogram at RTs of 1.69 and 2.01 min, respectively, with a base peak *m*/*z* of 214.0984 (average value, $n = 10$), corresponding to the addition of a formyl group to the aforementioned desulfonated moiety. An elemental composition of $C_{12}H_{12}N_3O$ was assigned to this product. The presence of formic acid in the UPLC eluents used may have led to the formation of this molecule as a by-product in the samples. However, it was not detected in either the abiotic or the heat-killed control samples, which were analyzed with the same UPLC eluents and gradient. At a RT of 2.98 min, a different peak with *m*/*z* of 278 was recorded, corresponding to formyl-SPY (P7), but its relative peak intensity remained below 5%. Similar formyl metabolites were predicted to be produced during the photodegradation of sulfadiazine in water,

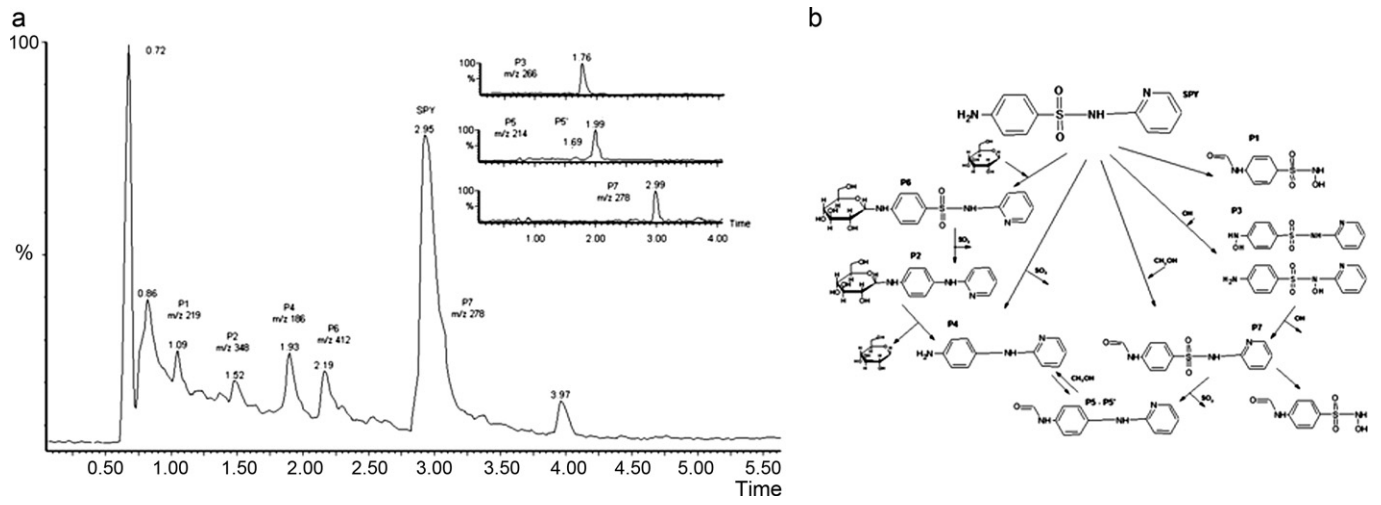

Fig. 3. Total ion chromatogram (TIC) of SPY degradation after 8 h in *in vivo* experiments (a) and proposed transformation products (b).

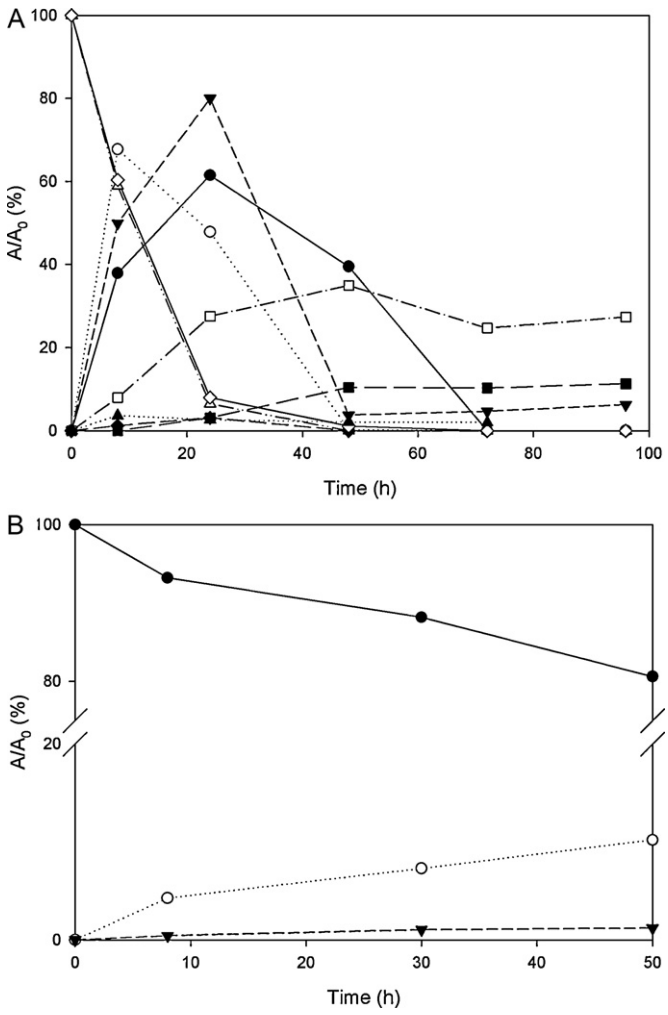

Fig. 4. Degradation of SPY over time and its major transformation products in *in vivo* experiments (A) and laccase experiments (B). Symbols (A): SPY (◊), *m/z* 219 (●), *m/z* 348 (○), *m/z* 266 (♦), *m/z* 186 (▼), *m/z* 214-1 (□), *m/z* 214-2 (■), *m/z* 412 (△), and *m/z* 278 (▲). Symbols (B): SPY (●), *m/z* 186 (○), and *m/z* 200 (▼).

the laccase-mediated degradation of SPY and during the degradation of SMZ by *T. versicolor* [16,22,33].

The loss of the pyridine ring and the addition of a hydroxyl group and a formyl group yielded a new product of *m/z* 219.0449 (*n* = 3) with an assigned elemental composition of $C_7H_{11}N_2O_4S$ (P1), which reached its maximum relative intensity after 24 h (61%). The hydroxylated moiety of SPY was also detected at a RT of 1.78 min with an observed mass of 266.0603 and an elemental composition of $C_{11}H_{12}N_3O_3S$ (P3). It was identified only in the samples taken after 8 h and 24 h and always at relative peak intensities <5%. Similar to SMZ degradation by *T. versicolor* [22], the OH group of the hydroxylated product was lost during MS/MS fragmentation.

3.3.2. Enzymatic degradation experiments

Although phenol-like compounds are the typical substrates of laccase-mediated oxidation, aromatic amines can also act as substrates [34]. As shown in Fig. 4, the decrease of SPY was significantly slower in laccase-mediated cultures than in the *in vivo* experiments, with a relative peak intensity of only 20% after 50 h. Two chromatographic peaks could be identified in the enzymatic degradation assays. The first peak appeared in the chromatogram at a RT of 1.16 min, with a base peak of *m/z* 200.0266 (average value, *n* = 3). The proposed elemental composition was $C_7H_8N_2O_3S$, which could correspond to a formyl intermediate (SI, Table S1). At a RT of 1.9 min, the desulfonated metabolite of SPY previously identified in the *in vivo* assays (*m/z* of 186.1052, *n* = 3; see Section 3.3.1) was also present. Although the relative peak intensity was lower in this case (10% maximum value), the presence of a common intermediate metabolite in the *in vivo* experiments supports the role of laccase in SPY transformation by *T. versicolor*. Mass spectral information could not be obtained for either of these degradation products primarily because they were present at low concentrations. As in Section 3.3.1, some of the predicted chemical structures were confirmed by obtaining the DBE values and detecting the sodium adducts.

3.4. Identification of degradation products from sulfathiazole

3.4.1. In vivo experiments

STZ was not fully degraded after 96 h of culture with the fungus. The glycosylated adduct and two transformation products were detected (Fig. 5).

The first signal in the chromatogram corresponded to N^4-glycosyl-STZ (P1), with an observed *m/z* of 418.0764 and an RT of 2.2 min. In contrast to N^4-glycosyl-SPY, which decreased during the reaction at rate similar to that of SPY, N^4-glycosyl-STZ decreased faster than STZ (Fig. 6). The STZ concentration started to decrease after 24 h, when 88% of the adduct had been used up by the fungus. This lag phase required by *T. versicolor* to start degrading STZ was not observed for SPY or SMZ [22], SAs that have 6-membered

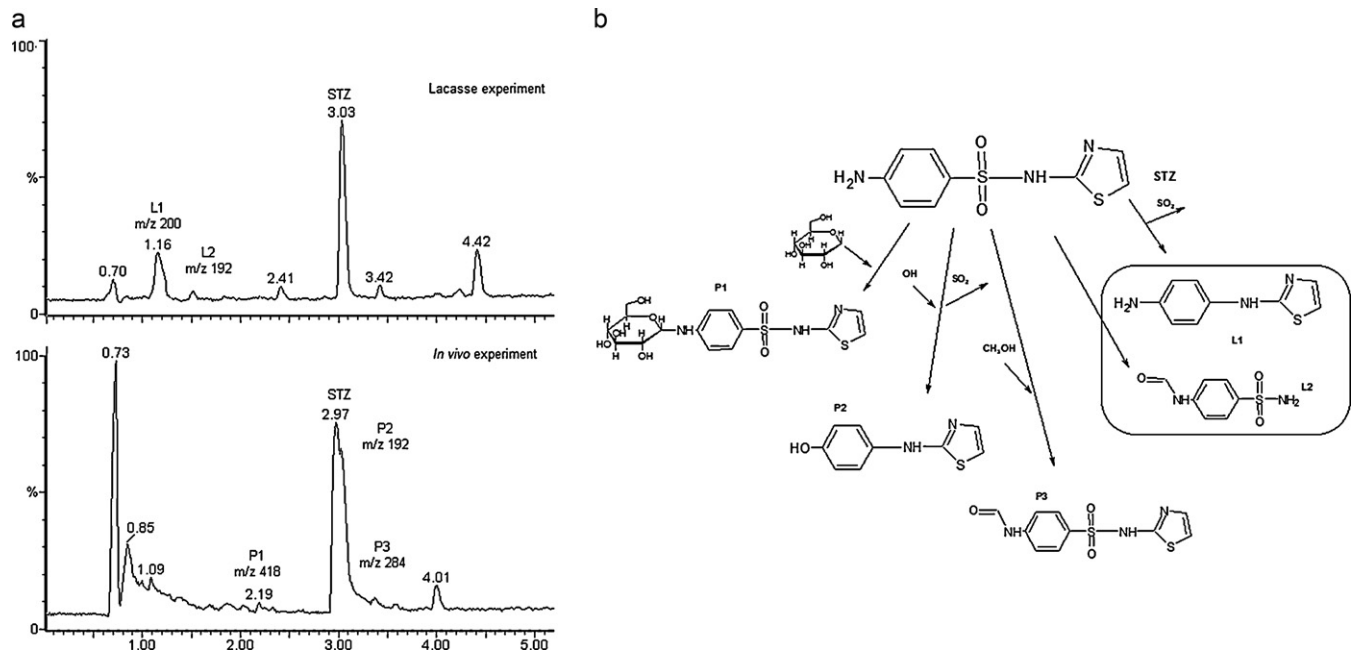

Fig. 5. Total ion chromatogram (TIC) of STZ degradation after 8 h in *in vivo* experiments (a) and proposed transformation products (b).

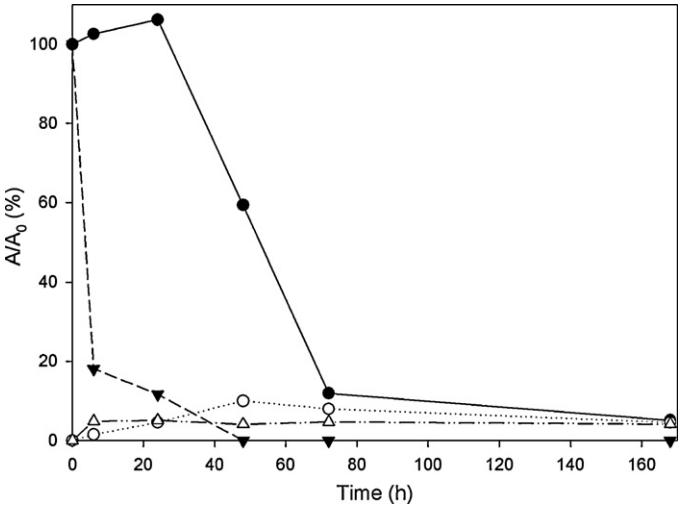

Fig. 6. Degradation of STZ over time and its major transformation products in *in vivo* experiments. Symbols: STZ (●), *m*/*z* 418 (▼), *m*/*z* 192 (△), and *m*/*z* 284 (○).

heterocyclic rings (pyridine and pyrimidine rings, respectively), suggesting that the substituent group of the SA secondary amine, in this case the 1,3-thiazole ring of STZ, determines the ability of the SA to be assimilated and/or degraded. The desulfonated product of the glycosylated adduct was not present for STZ.

At a RT of 2.99 min, a new peak was present in the chromatogram (Fig. 5a), with a base peak of *m*/*z* 192.0359 (average value, $n = 3$) and an elemental composition of $C_9H_8N_2OS$ (P2), which could correspond to the desulfonated and hydroxylated moiety of STZ, although the hydroxylated moiety of STZ was not detected in either the *in vivo* or laccase assays. The predicted molecular structure is shown in SI, Table S2. The normalized area was never higher than 5%. At a RT of 3.23 min, an additional signal with a base peak of *m*/*z* 284.0172 was identified as formyl-STZ (P3), which was at its highest concentration after 48 h of incubation. Upon applying a CE of 25, the product ion spectrum obtained showed two main stable fragment ions of *m*/*z* 120.0450 and *m*/*z* 100.0101 (SI, Fig. S1).

3.4.2. Enzymatic degradation experiments

We further investigated only two samples from the enzymatic degradation experiments, taken after 30 h and 50 h. Therefore, the trends over time for the intermediate products could not be determined. The desulfonated moiety of STZ was only detected in the laccase experiments. This peak appeared at a RT of 1.61 min, with an observed mass of 192.0584 and a corresponding elemental composition of $C_9H_{10}N_3S$. The signal intensity for this metabolite was similar for both samples (30 h and 50 h). As with SPY, the enzymatic degradation of STZ generated a compound with an observed *m*/*z* of 200.0257 and a proposed elemental composition of $C_7H_8N_2O_3S$ (L2, Fig. 5). The intensity of the signal seemed to decrease slightly between 30 h and 50 h.

3.5. Continuous degradation of SAs in a fluidized bed reactor

The promising results that we obtained at flask-scale, combined with previous studies that demonstrated the ability of *T. versicolor* to degrade SMZ [22], encouraged us to assess the simultaneous removal of the three antibiotics (SMZ, SPY and STZ) at the reactor scale. The performance of the reactor is shown in Fig. 7. The first stage (I) consisted of a batch setup reaction, during which complete removal of the SAs was accomplished. In this case, STZ was the last antibiotic to be fully depleted. To reduce the time of treatment, the continuous stage (II) was switched on with a HRT of 48 h when the glucose levels reached 1 g L^{-1} (at day 4). From this time on, glucose was supplied at the consumption rate to maintain a concentration of approximately zero. After four HRTs (a steady state was assumed after three HRTs), the degradation stabilized at 35% to 55% for the three SAs and SPY exhibited the highest removal. By the 12th day of operation, an excess in the growth of the biomass required us to purge the equivalent to nearly a third of the previous working volume so that the system could regain proper fluidization. Strategies such as purging and partial biomass renovation have been successfully applied to overcome operational issues and to achieve long-term continuous degradation in similar reactors such as those that use *T. versicolor* for decolorization [35]. The period of time during which the system was refilled to the original volume level and then stabilized at a HRT of 48 h is indicated in Fig. 7 as the

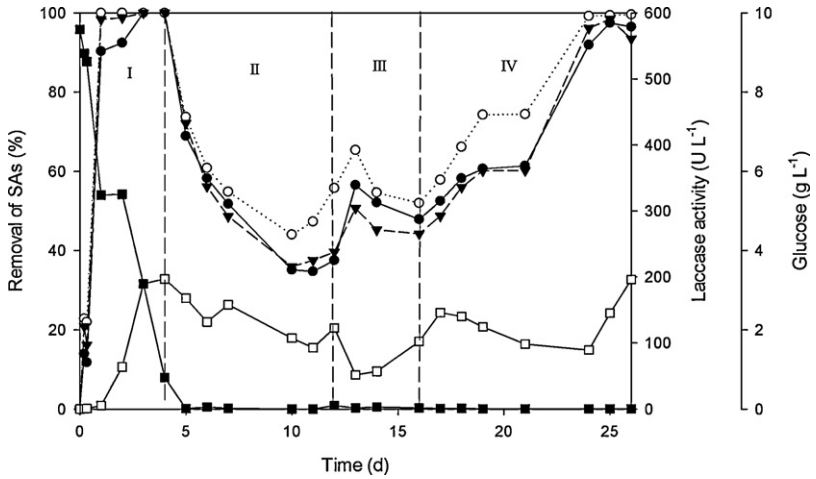

Fig. 7. Elimination of STZ (●), SPY (○) and SMZ (▼) (5 mg L^{-1} each) during treatment with *T. versicolor* pellets in a fluidized bed reactor. Stages of treatment: initial batch (I); continuous treatment, HRT 48 h (II); transition period (III); and continuous treatment, HRT 72 h (IV). Laccase activity (□) and glucose (■).

transition period (III). In an attempt to increase the removal efficiency, the HRT was changed to 72 h (IV). The elimination amount was significantly improved, reaching values of greater than 94% for all of the SAs after three HRTs. The laccase activity was monitored, and the activity values ranged from 100 U L^{-1} to 200 U L^{-1} during the continuous stages (II and IV). The simultaneous transformation of diclofenac, ibuprofen and naproxen has been accomplished in a fed-batch reactor containing *Phanerochaete chrysosporium* pellets [36]; however, reports of continuous depletion are uncommon and include the removal of textile dyes by *T. versicolor* [20,37] and endocrine-disrupting compounds by immobilized laccase [38].

4. Conclusions

The degradation of SPY and SMZ by *T. versicolor* has been demonstrated in this work. Despite the high concentrations used in the assays, SPY was fully degraded after the experimental time in the fungal cultures, and 7 different degradation products were elucidated, including formylated and hydroxylated products. STZ was more resilient toward degradation, and 12% of the initial concentration was still present after 96 h of incubation with the fungus in the *in vivo* assays, in which three different intermediate products were present. The respective desulfonated moieties and a common degradation product of *m*/*z* 200 were detected in the purified laccase experiments for both SAs. A continuous fluidized bed reactor with fungal pellets successfully eliminated a mixture of three SAs at a HRT of 72 h. Although degradation was accomplished and many metabolites disappeared or remained at very low concentrations at the end of the batch experiments, a toxicity assessment should be performed to determine any potential increase in the toxicity before a real application of *T. versicolor* for the removal of SAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2012.02.008.

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