Chemosphere 136 (2015) 9–19

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Non conventional biological treatment based on *Trametes versicolor* for the elimination of recalcitrant anticancer drugs in hospital wastewater

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HIGHLIGHTS

- Removals from 48 till 100% of anticancer drugs in hospital effluent treated by fungi.
- Cyclophosphamide and Ifosfamide remained unalterable whereas Tamoxifen was totally removed.
- Two hydroxilated positional isomers of Tamoxifen detected for first time in treated effluents.

ARTICLE INFO

Article history:
Received 8 December 2014
Received in revised form 16 March 2015
Accepted 24 March 2015
Available online 22 April 2015

Handling Editor: Jörg E. Drewes

Keywords:
Cytotoxic
Anticancer drugs
Hospital effluent
Removal
Trametes versicolor
HRMS

ABSTRACT

This work presents a study about the elimination of anticancer drugs, a group of pollutants considered recalcitrant during conventional activated sludge wastewater treatment, using a biological treatment based on the fungus *Trametes versicolor*. A 10-L fluidized bed bioreactor inoculated with this fungus was set up in order to evaluate the removal of 10 selected anticancer drugs in real hospital wastewater. Almost all the tested anticancer drugs were completely removed from the wastewater at the end of the batch experiment (8 days) with the exception of Ifosfamide and Tamoxifen. These two recalcitrant compounds, together with Cyclophosphamide, were selected for further studies to test their degradability by *T. versicolor* under optimal growth conditions. Cyclophosphamide and Ifosfamide were inalterable during batch experiments both at high and low concentration, whereas Tamoxifen exhibited a decrease in its concentration along the treatment. Two positional isomers of a hydroxylated form of Tamoxifen were identified during this experiment using a high resolution mass spectrometry based on ultra-high performance chromatography coupled to an Orbitrap detector (LTQ-Velos Orbitrap). Finally the identified transformation products of Tamoxifen were monitored in the bioreactor run with real hospital wastewater.

1. Introduction

Cancer is ranked (year 2012) in the second place (21%) of non-communicable diseases (this means non-infectious and non-transmissible medical conditions) which are causing deaths, after cardiovascular illness (48%) and followed by respiratory diseases (12%) (www.who.int); for that reason the high consumption of the drugs for chemotherapy treatments has became a cause of concern. These specific drugs have been shown to have potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor and/or teratogenic effects in several organisms, since they have been designed to disrupt or prevent cellular proliferation, usually by interfering in DNA synthesis or disrupting the endocrine system. The occurrences of these drugs in the aquatic environment could be especially critical since they are intrinsically hazardous. Several ecotoxicological studies have shown that in some cases such as for the cancer drug 5-Fluorouracil, the lowest observed-effect concentration values (in algal and bacterial assays) were close to the concentration found in sewage effluents (Zounkova et al., 2007). More recently, studies have revealed that mixtures of anticancer drugs in real samples possess an important toxicological effect comparing with the individual drug (Mater et al., 2014).

In general these so-called anticancer drugs can be released to the aquatic environment via hospital or domestic wastewater

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http://dx.doi.org/10.1016/j.chemosphere.2015.03.051
0045-6535/

(Kovalova, 2009; Kosjek and Heath, 2011; Ferrando-Climent et al., 2013, 2014; Negreira et al., 2013) since there is a large number of them not removed from the wastewaters neither by biological conventional treatments (Kümmerer et al., 1997; Ferrando-Climent et al., 2014) nor by advanced technologies studied so far, such membranes bioreactors (Lenz et al., 2007; Kovalova et al., 2012), electrolysis, and advanced oxidation processes (ozonization, UV, H_2O_2) (Chen et al., 2008; Zhang et al., 2013). Therefore there is a need of development and application of new technological alternatives for wastewater treatment, and for the removal of the anticancer drugs from sewage.

In this work the performance of an alternative biological treatment based on white-rot fungi (WRF) was explored to eliminate selected anticancer drugs. *Trametes versicolor* has been already shown to have a special capacity to remove a wide amount of pharmaceutical compounds (Cruz-Morató et al., 2013) including β-blockers, antibiotics, anti-inflammatory and psychiatric drugs and achieving even the mineralization of some compounds such Diclofenac and Ketoprofen (Marco-Urrea et al., 2010a,b,c,d; Prieto et al., 2011; Jelic et al., 2012; Rodriguez-Rodriguez et al., 2012). WRF has an unspecific oxidative enzymatic system which includes lignin-modifiers enzymes, in particular laccase and peroxidases (extra-cellular enzymes), and also intracellular enzymatic complexes (cytochrome P450) (Asgher et al., 2008). Hydroxylation, formylation, deamination and dehalogenation mechanisms in the anthropogenic pollutants take place during the fungi metabolism (Harms et al., 2011; Cruz-Morató et al., 2012) and enable the degradation of the parent compound. However, detoxification does not necessarily occur since transformation products (TPs) of parent compounds can be in occasions more recalcitrant or even more toxic than the parent compound.

The objective of this work was to study the potential ability of WRF *T. versicolor* to eliminate selected anticancer drugs from real hospital effluents. 10 anticancer drugs, selected because of their use, ubiquity, non-biodegradability and also their potential bioaccumulation in the environment (Besse et al., 2012), were monitored along the experiment performed in a fluidized bed bioreactor. Further individual degradation experiments were performed for Cyclophosphamide, Ifosfamide and Tamoxifen in order to assess their possible degradation by this fungus under optimal growth conditions and to identify transformation products generated in the experiments by high resolution mass spectrometry (HRMS). These three compounds were selected as target pollutants for individual studies due to their ubiquity in wastewater, low biodegradability as well as high toxicity.

2. Materials and methods

2.1. Fungus preparation

T. versicolor (ATCC#42530) was provided by the American Type Culture Collection. It was kept by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 d. *T. versicolor* was grown in form of pellets as previously described (Blánquez et al., 2004) and subsequently the pellets were washed with sterile deionized water before its use.

2.2. Standard preparation and reagents

Ciprofloxacin HCl, Cyclophosphamide, Ifosfamide, Methotrexate, Azathioprine, Etoposide, Docetaxel, Paclitaxel, Vincristine Sulphate and Tamoxifen Citrate were purchased by European Directorate for the Quality of Medicines and Healthcare (EDQM) Reference Standards (Strasbourg, France). Isotopically labeled compounds, used as internal standards, [^{2}H4]-Cyclophosphamide, [^{13}C6]-Tamoxifen Citrate, [^{2}H3]-Etoposide, [^{2}H3]-Methotrexate, [^{2}H3]-Vincristine Sulphate, [^{13}C4]-Azathioprine were purchased from Toronto Chemical Research Inc. (Canada) and [^{2}H8]-Ciprofloxacin from EDQM Reference Standards (Strasbourg, France). HPLC-grade Water and HPLC-grade acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany). Reagents like Formic acid 98% (HCOOH) were provided by Sharlab (HPLC-grade). Ethylenediaminetetraacetic acid disodium Salt 0.1 M solution (SV) and NH_3 30% was provided by Panreac (Barcelona, Spain).

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA). Glass fiber filters (1 μm) and nylon membrane filters (0.45 μm) were purchased from Whatman (U.K.). Glucose, ammonium tartrate dibasic and malt extract were purchased from Sigma–Aldrich (Barcelona, Spain).

Individual stock standard solutions of each target compound were prepared on a weight basis in methanol at 1 mg mL^{-1} and kept frozen at −20 °C. A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. Stock solutions of internal standards were also prepared in methanol and were stored at −20 °C. A mixture of these internal standards was also prepared by diluting the individual stock solution in methanol.

2.3. Hospital wastewater samples

The main hospital of Girona, Dr. Josep Trueta, was selected for this study. This municipality, which is located in the north of Spain, has approximately 96.000 habitants and the hospital, which counts with around 400 beds, receives the major of the oncologic patients of this area. Two non-consecutive samplings (Sample 1 and 2) were performed at hospital wastewater effluent prior to the connection with the wastewater treatment plant (WWTP).

In order to isolate the effect of *T. versicolor* onto the pollutants so discarding the activity from the rest of microorganism present in the wastewater, two treatments were tested in the wastewaters: Sample 1 was sterilized previous to all the experimental set-up while Sample 2 was not sterilized.

2.4. Biodegradation experiments

2.4.1. Degradation of anticancer drugs in bioreactors fed with real hospital effluents

A glass fluidized bed bioreactor with a working volume of 10 L (Blanquez et al., 2008) was used to carry out both sterile (Sample 1) and non-sterile (Sample 2) hospital wastewater treatment in batch mode. Approximately, 2.0 g dry weight (d.w.) pellets L^{-1} were inoculated in both sterile and non-sterile treatments. Fungal biomass was maintained fluidized by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 s open, 5 s close) and the air flow was 12 L h^{-1}. The bioreactor was equipped with a pH controller in order to keep pH at 4.5 and the temperature was maintained at 25 °C. Glucose and ammonium tartrate were fed continuously from their stock solution (300 g L^{-1} and 675 mg L^{-1}, respectively) at a flow rate to ensure an uptake rate of 0.31 g glucose g^{-1} d.w. pellets d^{-1} and 2 mg ammonium tartrate g^{-1} d.w. pellets d^{-1}. For sterile conditions the bioreactor and the wastewater (Sample 1) were autoclaved at 121 °C for 30 min. Samples of 250 mL were taken periodically. All the samples were filtered with 0.45 μm filters. 200 mL were stored at −20 °C to be further analyzed by UPLC coupled to a triple quadrupole-ion trap mass spectrometer (QqLIT). 50 mL from each sample were used to measure glucose concentration, COD, N—NH_4^+ and laccase.