Biodegradation of tetracycline by two basidiomycetes (pleurotus ostreatus and tra...

Business, Industries

Abstract

Todays, widespread use of antibiotics in modern medicine has been considered as a hot environmental topic. Tetracycline as over the counter therapeutic drugs, has aroused much public health attention. The most important following threat is easily spread of resistant bacteria through different routes. It is almost as a result of not completely metabolized drug via excretion to the wastewater. To avoid tetracycline release into land and food chain finding a suitable method which is capable of its degradation before entering to the environment can be affordable. In this way, a study was conducted to compare the biodegradability of tetracycline by two white rot fungi (P. ostreatus and T. versicolor) in liquid medium. Fungal laccase and peroxidase activity as involved degrading agents were also monitored during the experiment.

Results showed P. ostreatus was capable of suppressing 50 µgml-1 tetracycline within earlier than 4 days to undetectable concentrations. Almost Complete removal of tetracycline at 100 µgml-1 was occurred within the first 4 days of incubation. Whereas in uninoculated treatments just negligible removal of 9% tetracycline at 100 µgml-1 up to the end of the experiment was detected. Evaluation of the feasibility use of T. versicolor also revealed a reduction from 50 and 100 µgml-1 at initial concentrations to a residual 20. 5 and 55. 7 µgml-1 tetracycline by the end of the assay (10d), respectively. Tetracycline also led to a stimulatory impact on laccase and manganese peroxidase yield by T. versicolor whereas acts as an inhibitor in secretion of both enzymes by P. ostreatus. Considering to low degradation

rate of tetracycline and also its high persistency in liquid medium, P. ostreatus can be suggested as a more comparative one for biodegradation purpose.

Keywords: Biodegradation, Tetracycline, Laccase, Peroxidase, Pleurotus ostreatus, Trametes versicolor

Introduction

Antibiotic discovery was one of the most striking advances in the history of medical sciences. This group of pharmaceuticals have revolutionized modern medicine by treating many incurable illnesses and saved countless lives (). Today, antibiotics have become victim of their own success. Some misconceptions in correct use of antibiotics such as public unawareness and self-medication have led to the emergence of antibiotic resistance and mystery diseases (Marti, et al. 2014; Kümmerer, 2009; Czekalski et al. 2012; Le Corre et al. 2012; Schwartz et al. 2006). This problem becomes more intense when there is no regulations and prohibition against the indiscriminate use of antibiotics in some countries. As environmental point of view, major amounts of these antibiotics excrete from the body without any undergoing metabolism (Kümmerer, 2003; Huang et al. 2001). Since the usual conventional wastewater treatment plants are not capable enough to remove these kind of compounds.

So their frequent detection will easily occur via discharge of treated wastewater to receiving surface waters and lands which may pose as a serious threats within the environment. (Radjenović et al. 2009; Kümmerer, 2009; Michael et al. 2013; Zhang et al. 2015; Dominguez et al. 2014). To

date some potentially toxicological impacts of on terresial organisms have made the environmental protection agency (EPA) to classify this group of pharmaceuticals as contaminants of emerging concerns (CECS). For example, toxicity testing of the median effective concentration (EC50) on exposed algae and bacteria to a number of antibiotics to be evaluated as low as <1 mgl-1().

Among main classes of antibiotics, tetracycline family compounds are the third most frequently low-priced prescribed and broad spectrum antibiotics which consumed for human and animals' therapeutic and non-therapeutic purposes (USDA, 2008a; USFDA 2015; Pereira-Maia et al. 2010). It has been reported about nearly 30% consumed tetracyclines remain unchanged after excretion. Like other group of antibiotics the release of unchanged form of tetracycline to the environment has raised the number of bacteria resilient to this class of antibiotic (22). Tetracycline constitute three major groups (tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC)) (Granados-Chinchilla, 2017). The half-lives of TC, CTC and OTC are predicted around 55-578, 44, and 17-46 days, respectively which confirms their longevity and persistence. Tetracyclines also are classified as non-volatile compounds with high water solubility and low log Kow which possess them a hydrophilic nature and make their easily migration within environmental matrices.

However the reactivity and stability of different types of tetracyclines are apart from each other and very dependent on biochemically characteristics of the input environment. All tetracyclines are strong pH dependent which

have three forms of chemically behaviors included positive (pH <3. 3), neutral (3. 3 7. 68) in different pH. It has been caused each group of tetracycline diversely behave from pharmacokinetic point of view (). Overally among all three substitutions, CTC and OTC are seem to be more reactive in the environment than TC (Daghrir et al. 2013). In addition, CTC has superior solubility and degradability, followed by OTC, then TC (Loftin et al. 2008; Teixido et al. 2012). Based on the stated behavior of tetracycline, considering to its alarmingly spread via environment and taking actions toward some methods that potentially contribute to their degradation is necessary.

Biodegradation as a cost effective and biologically mediated technique always has been the most preferable used method to prevent the discharge of contaminants from their major sources. Among biodegradative agents, white rot fungi are classified as a group of Wood-decaying basidiomycetes which are extensively studied organism capable to degrade organic pollutants (Asgher et al. 2008; Torres et al. 2003; Ahn et al. 2002, Badia-Fabregat et al. 2014; García-Galán et al. 2011; Llorens-blanch et al. 2015; Yang et al. 2013; Cvančarová et al. 2012). The involvement of these fungi in such degradative processes can be efficiently related to secrete some extracellular enzymes (mainly laccase, lignin peroxidase, manganese peroxidase). The activity of these enzymes represent the oxidative behavior of white-rot fungi in the media which has aroused interest in using the pure forms of them in bioremediation (Bollag et al. 2003). As today commercial laccase is extracted from Trametes versicolor, one of the most well-studied

white rot fungi. However the role of these ligninolytic enzymes is scarcely investigated in degradation of antibiotics.

So to achieve this aim, a batch test was conducted to evaluate and compare the degradative capability of two white rot fungi (P. ostreatus and T. versicolor) in exposed mediums to tetracycline hydrochloride (50 and 100 µgml-1). Fungal Biodegradation of tetracycline was examined for 10 days period time. The experimental levels of tetracycline were chosen not likely as occurring concentrations in the environment, to enable detection of the differences more strongly. During the experiment, changes in laccase and peroxidase enzymatic activity, fungal secreted protein and biomass were also measured in three time intervals (4, 7 and 10 days). In order to assess possible correlation between tetracycline biodegradation with these factors.

Materials and methods

Chemicals and reagents

Tetracycline hydrochloride (TC) (≥95%, CAS 64-75-5), ABTS (2, 2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were purchased from sigma-aldrich (CHEMIE GmbH, Germany). 2, 6-DMP (2, 6-dimethoxyphenol) (99. 0%) was supplied by the ACROS organics, New Jersey, USA. HPLC grade methanol (MeOH) and acetic acid (AA) were obtained, from Merck (Darmstadt, Germany). Solid reagents were purchased from Merck and included Sodium acetate trihydrate, malonic acid, citric acid, potassium dihydrogen phosphate, ethylenediaminetetraacetic acid disodium salt (EDTA), and sodium hydroxide (NaOH). All reagents were analytical-reagent grade.

Organisms

The fungal strains (Pleurotus ostreatus & Trametes versicolor) were purchased from Fungi Perfecti, Olympia, Washington as plug fungal spawns.

Culture conditions

Stock solution of tetracycline were prepared at concentration of 2500 µgml-1 in methanol. The experimental concentrations (50 and 100 µgml-1) of tetracycline appropriately prepared by dilution of initial stock solution. Then added to each treatment flask up to final concentrations of 50 and 100 µgml-1. In order to have fresh fungal colonies, 0. 5mm2 agar plugs subcultured periodically on malt extract agar (MEA) plates. Six-day-old fungi colonies grown on MEA plate at 30 °C were used to inoculate the flasks. Mycelium plugs (1 cm2) were aseptically cut with conical piping tips from actively growing zones were inoculated in 20ml potato dextrose broth (PDB) medium in a 50ml Erlenmeyer flask. All flasks covered with aluminum foil to prevent photodegradation and incubated at 30 °C. The optimal temperature was set according to the maximum laccase and peroxidase production in the dark (Zhang et al. 2011; Kümmerer, 2009). As cultivation at temperatures higher than 30°C reduce the activity of laccase and peroxidase.

Experimental design: (experiment setup)

To assess tetracycline (TC) biodegradation, a batch experiment was planned in a completely full factorial design using three replicates. Experimental factors were included: Separately inoculated two fungal strains (P. ostreatus and T. versicolor) and three concentrations of tetracycline (0, 50 and 100 µgml-1). Furthermore to analysis the stability of tetracycline during the test

period, a reference medium was exposed to 100 µgml-1 of tetracycline. Samples from this medium were used to remove time and matrix effect on degradation measurements during three different time intervals without fungal inoculation. After 4, 7 and 10 days from the beginning of the trial, samples from each treatments were collected and stored in -20°C for later analytical assays. Data analysis: Statistical analyses were performed using the JMP 13 software.

Biomass and protein determination

Biomass growth was measured as mycelium dry weight by vacuum filtering and dry at 70°C to reach constant weight. Total Protein in liquid culture was determined by the Bradford method (Biorad protein assay) using bovine serum albumin as the standard at 595 nm wavelength.

Enzyme activity assays

Ligninolytic enzymes were analyzed in the culture medium after centrifugation at 5, 000 g for 5 min. Laccase activity was assayed by monitoring the oxidation of ABTS (2, 2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as described by Bourbonnais and Paice (1990) in 0. 1M sodium acetate buffer pH 4. 5 and the absorbance of each sample was taken after a 1. 5 min time interval at 420 nm (). An extinction coefficient of 36000 M-1cm-1 was used.

MnP activity was determined spectrophotometrically at 468 nm by use of 2, 6-dimethoxyphenol (DMP) as the substrate and following the formation of Mn3+ −malonate complex at pH 4. 5 in 50mM sodium malonate buffer with 1mM MnSO4. The reaction was initiated by adding H2O2 to the final

concentration of 1mM (Wariishi et al. 1992). The reaction was followed for 1. 5 min at room temperature. Changes in Absorbance per min was converted to U. L-1 using an extinction coefficient of 49600 M-1cm-1.

Enzyme results were expressed as activity units (U) per min. One U was defined as the number of micromoles of ABTS and DMP oxidized per min. Specific enzyme activity also was expressed as U. mg-1 total protein in liquid medium. To compare the effect of tetracycline addition on the specific laccase and peroxidase activity of the fungi, the activation factor, KEA, was defined as the ratio of stimulated specific enzymes activity (ESA) related to basal specific enzymes activity (EBA).

Extraction and clean up

EDTA-McIlvaine buffer (0. 1 mol. L-1, pH 4. 0) was prepared by dissolving 21. 01 g of citric acid monohydrate, 44. 78 g of Na2HPO4·12H2O and 60. 5g of Na2EDTA·2H2O in 1. 625 L of water. 10ml sample transferred to 50mL polypropylene centrifuge tubes. 10mL of EDTA-McIlvaine buffer were added to each tube. The tubes were capped, vortex-mixed for 30s, and then shaken for 20 min in 120rpm. Samples were centrifuged at 3000rpm for 5 min. The supernatant layer was separated.

OASIS HLB (Waters Corporation) 6mL 200mg solid phase extraction (SPE) columns preconditioned consecutively with 3 mL of methanol and 3 mL of water were used for the clean-up procedure. The above sample solutions were allowed to pass through the columns at a rate of approximately 1mL. min-1. HLB columns were washed with 3mL of water, then vacuum dried. The SPE cartridges were vacuum-dried for 5 min, and the analytes were eluted

with 3mL of methanol. The eluate was evaporated to dryness with gentle nitrogen flow and then redissolved in 1mL of methanol. The reconstituted samples were filtered into auto-sampler vials using 0. 22 µm syringe filters for LC-MS/MS analysis.