

BIODEGRADATION OF BISPHENOL A DURING SUBMERGED CULTIVATION OF *TRAMETES VERSICOLOR*

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ABSTRACT

Bisphenol A is a persistent chemical, which is widely distributed in the environment despite its short half-life. The continuous release of BPA may cause a chronic exposure of the organisms during sensitive life stages. The chemical possesses not only endocrine disrupting functions but also by oxidative stress may cause damage to the liver cells. In this study the ability of *Trametes versicolor* 8979 to degrade Bisphenol A during submerged cultivation was examined. The chemical was introduced to the medium in 500ppm concentration and the laccase and MnP activities, as well as the residual BPA concentration were monitored. The strain was able to remove completely Bisphenol A from the medium in 6 hours.



Keywords: *Trametes versicolor*, Bisphenol A, Laccase, Biodegradation

INTRODUCTION

Bisphenol A (4,4'-isopropylidene-2-diphenol) is a synthetic compound that is formed by the condensation of phenol with acetone. It is widely used in the production of multitude of products including building materials, electronic components, long-lasting resistant to heat food containers and many widely used plastic products (Staples *et al.*, 1998, Jiao *et al.*, 2008, Björnsdotter *et al.*, 2017). The annual production of BPA in 2016 was 8 million metric tons and it's assumed to hit over 10 million metric tons by 2022 (Bisphenol, 2016). BPA polymerization leads to formation of compounds which could be hydrolyzed under aggressive conditions such as high temperatures and extreme pH values, thus causing BPA leaching into the environment. (Chouhan *et al.*, 2014). The average daily BPA exposure was evaluated to be from 0.48 to 1.6 µg/kg body mass/day, for adults and children, respectively (Flint *et al.*, 2012).

Bisphenol A is a persistent chemical, which is widely distributed in the environment despite its short half-life. (Oehlmann *et al.*, 2009). Release can occur during chemical manufacture, transport, and processing. Post-consumer releases are primarily via effluent discharge from municipal wastewater treatment plants, leaching from landfills, combustion of domestic waste, and the natural breakdown of plastics in the environment (Crain *et al.*, 2007; Kang *et al.*, 2007; Kinney *et al.*, 2006; Sidhu *et al.*, 2005; US Environmental Protection Agency, 2010). Due to BPA environmental ubiquity and the wide usage of compounds' analogues, the possibility of chronic rates of exposure of the organisms escalates. Also the exposure could happen in sensitive life stages such as infant development (Flint *et al.*, 2012). BPA is known to possess endocrine disrupting activity and could cause damage of liver cells with the mechanisms of oxidative stress. Studies show the possibility of BPA to modulate the immune activity, also demonstrate the possession of mutagenic and teratogenic activity on eukaryotic cells (Michalowicz, 2014). BPA exposure in early stage of development leads to increased risk of developing mammary and prostate cancers (Seachrist *et al.*, 2016, Noszczyńska and Piotrowska-Seget, 2018). The exposition of the fetus, infants and young children to BPA is considered to be especially harmful due to the lack of information regarding hormones' activity, synthesis and elimination. Higher levels of anxiety, hyperactivity, depression and conduct problems could be linked with BPA exposure during childhood development (Rykowska and Wasiak, 2006, Braun *et al.*, 2009, Ejaredar *et al.*, 2017).

The growing problem on Bisphenol A release into the environment and their toxic effects on humans and wildlife require the elaboration of new technological solutions able to minimize or eliminate the environmental exposure (Cabana *et al.*, 2007). Some of the successfully developed methods for BPA removal are based on biological or electrochemical oxidation, ozonation and biodegradation (Kang *et al.*, 2006, Yang *et al.*, 2013). The usage of microorganisms for BPA removal is promising method regarding waters. Biodegradation techniques allow

not only the removal of persistent pollutants but also are considered promising in the field of toxicological risk reduction not only of BPA but for other bisphenols as well (Sakai *et al.*, 2007, Danzl *et al.*, 2009, Zhang *et al.*, 2013, Noszczyńska and Piotrowska-Seget, 2018). Several bacteria distributed in waste water treatment plant as well as in river water are capable to easily degrade BPA. Usually the degradation process is initiated not by single microorganism but by whole microbial communities. Still degradation studies are focused more on the isolation and characterization of single microorganisms and their biodegradation abilities. However, the practical application of such experiments is limited due to a lack of efficacy at high concentrations (Xiong *et al.*, 2017b, Noszczyńska and Piotrowska-Seget, 2018).

In recent years the ability of white-rot fungi to degrade harmful chemicals has been widely investigated. Those basidiomycetes possess lignin-degrading activity presented by the enzymes laccase, manganese-dependent peroxidase (MnP) and lignin peroxidase (LiP). It has been reported that MnP and laccase from *Pleurotus ostreatus* are able to degrade BPA and have the ability to ameliorate or eliminate the estrogenic activity of BPA (Brugnari *et al.*, 2018; Hirano *et al.*, 2000, Lee *et al.*, 2005). Moreover, studies on laccases of the basidiomycetes *Trametes villosa* (Fukuda *et al.*, 2001), *Coriolopsis polyzona* (Cabana *et al.*, 2007), *Trametes versicolor* (Kim *et al.*, 2008; Margot *et al.*, 2013, Zeng *et al.*, 2017), *Trametes polyzona* (Chairin *et al.*, 2013), *Grifola frondosa* (Nitheranont *et al.*, 2011), as well as laccases from other basidiomycetes (Tanaka *et al.*, 2000; Kim and Nicell, 2006), revealed the potential of these oxidative enzymes for BPA degradation. Despite the lower redox potential of laccases, they are able to oxidize non-phenolic compounds when low molecular mass mediators are present. This makes them suitable for industrial and environmental purposes (Cañas and Camarero, 2010; Camarero *et al.*, 2014 Daassi *et al.*, 2016). This study focuses on investigation of degradation ability of *Trametes versicolor* 8979 towards BPA during submerged cultivation in the presence of BPA.

MATERIAL AND METHODS

Fungal strain

Trametes versicolor NBIMCC#8979 was isolated from the hills in Plovdiv, Bulgaria and deposited in the National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. The strain was maintained on Chapek Dox agar, containing sucrose – 30g/L; yeast extract – 5g/L; NaNO₃ – 2g/L; K₂HPO₄ – 1g/L; MgSO₄ – 0.5g/L; KCl – 0.5 g/L, FeSO₄ – 0.01g/L and agar – 15g/L with final pH 6.5. The cell culture was grown on agar plates for 7 days, then was stored at 4 °C.

Inoculum. Culture conditions. Biodegradation of BPA

For the inoculum preparation a 7-day old plate culture was used. The inoculation was carried out with 5 % spore suspension inserted to a 300 ml Erlenmeyer flask containing 100 ml Czapek-Dox medium containing: sucrose – 30g/L; yeast extract – 5g/L; NaNO₃ – 2g/L; K₂HPO₄ – 1g/L; MgSO₄ – 0.5g/L; KCl – 0.5 g/L and FeSO₄ – 0.01g/L. The pH of the media was adjusted with 0.1M HCl to 6.5 prior the sterilization. The inoculated flasks were incubated at 28 °C and 220 rpm until the end of exponential phase of growth. Bisphenol A was added to the cultural medium in dry form in final concentration of 500 ppm after which the incubation continues at the same conditions. The control sample contained uninoculated Chapek-Dox media and 500 ppm BPA.

Samples were taken every hour for the first 6 hours and after 24 hours. The biomass and protein concentration were determined, as well as the laccase and manganese-dependent peroxidase activity and residual Bisphenol A concentration.

Enzyme assays

Laccase activity was determined using syringaldazine as substrate. The reaction mixture contained cultural broth, 50mM potassium phosphate buffer (pH4.5) and 0.216 mM substrate solution in methanol. The change of absorption values at 530 nm was monitored at 37°C for 5 minutes (Ride, 1970). One unit of enzyme activity corresponds to 0.001 change in the absorbance at the reaction conditions and it is expressed in units per mL.

The activity of the manganese-dependent peroxidase (MnP) was determined by measuring the oxidation of Mn (II) to Mn (III) at 270 nm. The reaction mixture contained 1.0 mM MnSO₄ in 50mM sodium malonate buffer (pH4.5) and the reaction was started by the addition of 0.5 mM H₂O₂ solution (Wariishi et al., 1989). One unit of enzyme activity is defined as the enzyme quantity required to oxidize 1µmol substrate for 1 minute at the reaction conditions.

Determination residual Bisphenol A concentration

Bisphenol A was extracted from the cultural medium by addition of an equivalent amount of acetonitrile (Merck KGaA) to the sample followed by ultrasound treatment for 30 minutes. An ultrasonic bath Siel (Siel Ltd.) with a frequency of ultrasound 45 Hz was used. Then the sample is treated with Carrez solutions (Carrez, 1909) to remove sugars and proteins and followed by centrifugation at 6000 rpm for 15 minutes. The precipitate was discarded and the supernatant was transferred to a pear-shaped flask and evaporated under vacuum at 40°C and 200 rpm on a rotary vacuum evaporator IKA RV10, equipped with a thermostatic water bath IKA HB 10 (IKA®-Werke GmbH& Co.) and vacuum-pump Ilmvac (Gardner Denver Medical) until dry. The sample was resuspended in 5 mL acetonitrile (HPLC grade) and filtered through 0.45 µm membrane filter. Aliquot of 20 µL was taken for chromatographic analysis.

The HPLC determination was performed at Agilent 1200 Infinity Series equipped with UV-detector. The separation column was Zorbax Eclipse PAH with 5µm particle size and 4.6 nm inner diameter and 150 mm length. Acetonitrile (HPLC grade, Merck) was used as mobile phase at flow rate of 1 mL/min. The detection was made at 220 nm. Each sample was injected three times and the mean was calculated.

BPA's derivatives were determined using GC Trace 3000 with TR-5MS column and single quadropole MS ISQ QD (Thermo Fisher Scientific Inc.). The sample (1 µL) was injected in split mode. The oven temperatures were as follows: 3 min 60 °C, increasing 15 °C/min to 310 °C and hold for 5 minutes. The transfer line temperature was 260 °C and 220 °C for the ion source. The flow rate of the helium, was 1 mL/min, 70eV, m/z 35-350.

RESULTS AND DISCUSSION

The basidiomycetes are proven to have the ability to degrade aromatic compounds due to the production of the lignin-degrading enzymatic complex. This complex plays an important role in the degradation of compounds with phenolic and polycyclic structure. *Trametes versicolor* 8979 produces high activities of laccase and manganese-dependent peroxidase which are two of the three enzymes, part of the lignin-degrading complex. The ability of *Trametes versicolor* 8979 to biodegrade bisphenol A during submerged cultivation was investigated. Laccase and manganese-dependent peroxidase activities are shown on figure 1. There is an induction of the both enzyme activities with the highest values measured at the 2nd hours after the BPA introduction in the media for the laccase and at the 3rd hour for the manganese-dependent peroxidase.

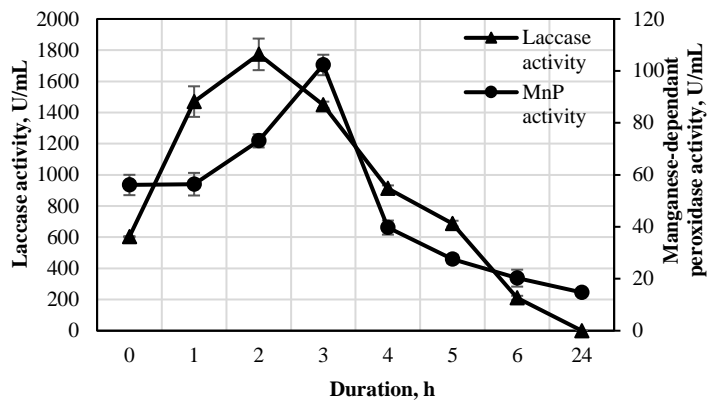


Figure 1 Enzymatic activity of laccase and manganese-dependent peroxidase during the biodegradation process

Changes in the concentration of BPA were noticeable with the analysis of the first sample at the first hour of the experiment. There was decrease in the concentration of nearly 50% after only one hour of interaction between the compound and the culture. The observed data is shown on figure 2. BPA residual concentration is below 100 ppm after 3 hours of degradation and on the 6th our represents only 1.28% of the initial concentration. There was no BPA residues detected at the 24th hour of the experiment.

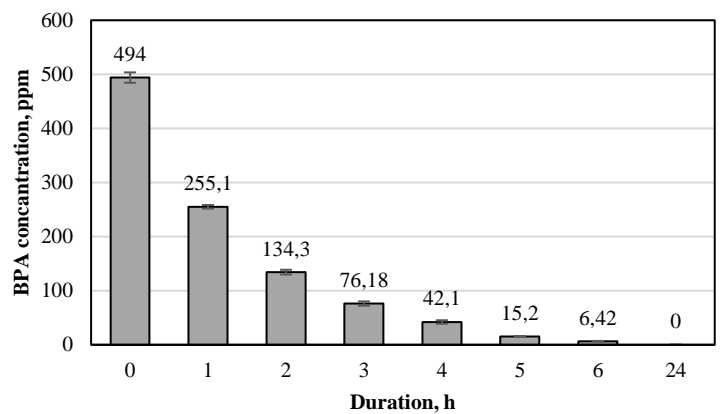


Figure 2 BPA concentration during the degradation process

Despite the limited knowledge regarding the BPA degradation with *Trametes versicolor* strains it is well known that the key role on this process is played by the enzymes, part of the lignin-degrading enzymatic complex. The increase of laccase activity in the first hours of the process (fig.1) correlates with intense BPA degradation (fig.2). The benzene rings in BPA structure are the main cause of the induction of laccase production during the experiment. Manganese-dependent peroxidase synthesis is also induced with the addition of aromatic compounds and organic acid in the medium. This induction of the enzymatic synthesis is correlating with the results, reported by Kim et al. (2008). The laccase gene expression was examined during degradation of endocrine-disrupting chemicals including BPA. It was proven that the introduction of BPA into the medium led to increase of the activity of both laccase and manganese-dependent peroxidase.

The BPA concentration during the process is following a classic 1st order reaction kinetics, which allows the calculation of the rate constant and compound's half-life. The rate constant is expressed as follows:

$$C_t = C_o \cdot e^{-kt} \quad (1)$$

where C_t is the BPA concentration at any time; C_o is the initial concentration and k is the rate constant. The determination of the rate constant depends on the compound's half-life and is expressed as follows:

$$k = \frac{\ln 2}{t_{1/2}} \quad (2)$$

Using those equations the rate constant and the compound's half-life were determined to be 1.386 h⁻¹ and 0.5h, respectively. The BPA biodegradation process is a result of the metabolism of the compound by the fungi, as well as the occurring oxidation processes by laccase and manganese-dependent

peroxidase. In this matter a direct correlation between the enzyme activities and the biodegradation could not be made, which is visible from the figures.

The results obtained after the GC-MS analysis showed that there are no typical BPA intermediate degradation products in the analyzed samples. Also there was no residual BPA detected under the HPLC limit of detection. The degradation of Bisphenol A with *Trametes versicolor* strain is proven to conclude with glycerol as a final product (Daassi et al., 2016). It is possible that BPA was fully degraded and mineralized by *Trametes versicolor* 8979. Such pathway where Bisphenol A degradation is full was proposed by Gasara et al. (2013). Other possibility is the formation of polymeric structure due to the laccase oxidative action which are separated during the sample filtration. In this matter it would be more accurate if the used term is removal rather than degradation of Bisphenol A. In this particular experiment the biodegradation is most likely a complex process leading to full bisphenol A removal from the samples.

CONCLUSION

Bisphenol A was completely removed from the medium during submerged cultivation of *Trametes versicolor* 8979. The strain possess high laccase and manganese-dependent peroxidase activities which are the main reason for the fast and effective process of bisphenol A removal. Moreover, the introduction of the chemical to the medium lead to induction of the enzyme activity. Bisphenol A was almost completely removed from the reaction media for 6 hours at 28°C and 220 rpm.

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