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ScienceDirect
Journal of Hazardous Materials 154 (2008) 33–37
Journal of Hazardous Materials
www.elsevier.com/locate/jhazmat

Impact assessment of bisphenol A on lignin-modifying enzymes by basidiomycete *Trametes versicolor*

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Received 31 May 2007; received in revised form 22 September 2007; accepted 24 September 2007
Available online 29 September 2007

Abstract

The impact of different concentrations of bisphenol A (BPA) was evaluated on growth of the white-rot basidiomycete, *Trametes versicolor*, and on the expression of genes encoding lignin-modifying enzyme (LME) activities. Effective doses (EDs) were obtained from fungal growth rate to monitor LME activities and the expression levels of their encoding genes. The fungus showed mycelial growth at concentrations of up to 300 μg ml^{-1} of BPA with an ED_{50} value of 185 μg ml^{-1}. The LME activities were stimulated by BPA concentrations up to 300 μg ml^{-1}. The lignin peroxidase (LIP) encoding gene may be sensitive to BPA stress.

Keywords: Bisphenol A (BPA); Effective dose (ED); Endocrine disrupting chemical (EDC); Lignin-modifying enzyme (LME); Semiquantitative reverse transcription-polymerase chain reaction (sqRT-PCR); *Trametes versicolor*

1. Introduction

A concern about the impact of endocrine disrupting chemicals (EDCs), especially endocrine disruption in humans and wildlife, has increased since the late 1990s [1]. Bisphenol A (BPA) is one of many high-production volume chemicals in the world widely used in plasticisers and epoxy resins which contaminate water and soil [2–4]. Recently, there has been increasing interest in the microbial degradation of EDCs. Lignin-modifying enzymes (LMEs) produced by white-rot fungi can catalyse polymerisation and precipitation of a broad range of natural and synthetic materials [5,6]. LMEs can be also used to break down and reduce the oestrogenic activity of BPA, due to the similarity of its chemical structure with that of lignin. The white-rot basidiomycete, *Trametes versicolor* used in this study has been previously investigated for application in biodegradation of toxic compounds, including BPA [7–10]. White-rot fungi possess potency as biologically based inexpensive elimination systems for use in EDC-contaminated sites, and commercial usage is expected to take place in the future. In our study, we selected LMEs to monitor the impact of EDC (BPA) in view of this potential future application.

The objective of this study was to assess the potential impact of BPA on the white-rot basidiomycete, *T. versicolor* for the production of LMEs. Initially, fungal growth rates were examined to evaluate fungal resistance to BPA stress. Effective dose data, $(ED)_{25}$, ED_{50} and ED_{75} were used for LME activity assays and quantification of transcript abundance for genes encoding LMEs.

2. Materials and methods

2.1. Reagents

BPA was purchased from Fisher Scientific (Loughborough, UK). All other chemicals used were of reagent grade and purchased from Sigma-Aldrich (Poole, Dorset, UK) or Fisher Scientific. All culture media and additives were supplied by Sigma–Aldrich and Fisher Scientific.

2.2. Organism and culture conditions

The white-rot basidiomycete *T. versicolor* R101 (Warwick HRI, Wellesbourne, Warwickshire, UK) was maintained in 7%

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doi:10.1016/j.jhazmat.2007.09.098

(final concentration) glycerol stock solution at −80 °C and used for all experiments. Glycerol stocks were thawed on ice before use and 100 μl of stock was spread on malt-extract agar (MEA) (OXOID: Fisher Scientific; 30 g malt extract, 5.0 g mycological peptone and 15 g agar per litre, pH 5.4 ± 0.2) in a 9 cm Petri plate and incubated for a few days at 25 °C. Five 6 mm diameter agar disks were taken and inoculated onto fresh MEA plates and incubated for 1 week at 25 °C.

The basal medium used for experimental cultures (nitrogen-limited medium: NL) was modified from Kirk et al. [11]. It contained 10 g glucose, 0.2 g ammonium tartrate, 1.0 g KH_2PO_4, 0.26 g $NaH_2PO_4{\cdot}H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.075 g $CaCl_2{\cdot}2H_2O$, 1 mg thiamine·HCl, 1.46 g dimethyl succinic acid and 10 ml trace element solution in 1 l deionised water at pH 5.0, sterilised by autoclaving (121 °C, 15 min). NL liquid was solidified with 1.5% Technical Agar No. 2 (OXOID: Fisher Scientific). Two agar plugs (6 mm) from the outer circumference of fungal mycelia growing on a NL plate (15 days) were used as the inoculum. The fungus was grown in 15-ml stationary cultures in the dark.

2.3. Growth rate

BPA was used to evaluate the impact on fungal growth rate and to obtain EDs for the following enzyme activity assays and molecular studies. In order to estimate how resistant the fungus was to the BPA stress, the higher concentrations of BPA, which might be irrelevant in the aqueous environment, were included in the study. A BPA stock solution in *N*,*N*-dimethylformamide (≥99%) (DMF: Sigma–Aldrich) was filtered through a 0.22 μm filter (Fisher Scientific) and then a dilution series of BPA was prepared in the sterilised NL liquid medium using the stock solution. An aliquot of each dilution was then added to the autoclaved NL agar (final concentrations at 0, 10, 50, 100, 200, 250, 300 and 400 μg ml^{-1} in 25 ml in total volume) and solidified at room temperature. In each treatment, the limit concentration of DMF was set at 0.5% (v/v) in agar, below the commonly used concentration for microbial bioassay. A 6-mm mycelium disc was placed in the centre of each treatment plate and the plates were incubated for 20 days at 25 °C in the dark. Fungal diameters were measured daily taking two measurements at right angles to each other. Four replicates were prepared for each treatment. The temporal radius was plotted and the linear regression used to determine growth rate (mm day^{-1}) with S.E.M. indicated by error bars.

2.4. LME activity assays

To study the impact of BPA on LME activities, two 6-mm agar disks were inoculated in 15 ml NL liquid medium in an EasyFlask™ (Nalge Nunc: Fisher Scientific) for 14 days at 25 °C, without shaking in the dark. Bottles were loosely capped to allow passive aeration. The BPA stock solution in DMF was used to prepare for a dilution series of BPA in the sterilised NL liquid medium at final concentrations of 0, 50, 200 and 300 μg ml^{-1}. In each treatment, the limit concentration of DMF was set at 0.5% (v/v) in liquid culture. Five replicates were prepared for each treatment. Culture fluid (2 ml) was taken from each treatment every 2 days, filtered through a 0.22 μm filter (Fisher Scientific) and stored at −20 °C for enzyme activity assays. Data points in all cases are means of five replicates, with S.E.M. indicated by error bars.

2.4.1. Laccase (LCC)

LCC activity was assayed spectrophotometrically with 2,2′-azino-bis-ethylbenthiazoline (ABTS) as a substrate [12]. A reaction mixture (200 μl) contained 0.1 M sodium acetate buffer (pH 5.0), 50 μl aliquots of culture fluid and 0.5 mM ABTS. The reaction mixture was incubated at 37 °C for 10 min, and oxidation of ABTS was measured by monitoring the increase in absorbance at 405 nm for 10 min in the kinetic mode. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidise 1 μmol ABTS per min using an extinction coefficient of 1.5471, which was calculated from the calibration curve. Enzyme activity was expressed as U ml^{-1} liquid medium.

2.4.2. Manganese peroxidase (MNP)

MNP activity was assayed spectrophotometrically using *N*,*N*,*N*,*N*-tetramethyl-1,4-phenylenediamine(-2HCl) (TMPD) as a substrate, initialised by addition of H_2O_2 [13]. A reaction mixture (250 μl) contained 0.65 M sodium tartrate buffer (pH 5.0), 0.65 mM $MnSO_4$, 50 μl culture fluid, 0.5 mM TMPD and 0.65 mM H_2O_2. The reaction mixture was incubated at 37 °C for 10 min and the increase in absorbance measured at 610 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidise 1 μmol TMPD per min using an extinction coefficient of 1.7288, which was calculated from the calibration curve. Enzyme activity was expressed as U ml^{-1} liquid medium.

2.4.3. Lignin peroxidase (LIP)

LIP activity was assayed spectrophotometrically using ABTS as a substrate, initialised by addition of H_2O_2 [14]. A reaction mixture (250 μl) contained 0.3 M citrate/0.4 M phosphate buffer (pH 4.5), 50 μl culture fluid, 0.4m M ABTS and 0.4 mM H_2O_2. The reaction mixture was incubated at 40 °C for 2 min and the increase in absorbance measured at 405 nm [15]. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidise 1 μmol ABTS per min using an extinction coefficient of 1.4846, which was calculated from the calibration curve. Enzyme activity was expressed as U ml^{-1} liquid medium.

2.5. Quantification of transcript abundance for genes encoding LMEs

2.5.1. Total RNA preparation

To study the impact of BPA on LME encoding gene transcription levels, two 6-mm agar disks were inoculated in to 15 ml NL liquid medium and incubated in the same conditions as those used for enzyme activity assays for 10 days. Triplicates were prepared for each treatment. Total RNA was prepared by a modification of the TRI reagent manufacturer's protocol (Sigma).

The mycelial mat was filtered through a 0.22 μm filter (Nalgene: Fisher Scientific) and then removed from the filter using sterilised tweezers. It was washed twice with sterilised water, then, immediately frozen in liquid nitrogen. The frozen mycelia from each bottle were separately ground to a fine powder with a cold, sterilised mortar and pestle. One millilitre of TRI reagent was added to homogenise the sample. It was then transferred to a 2 ml sterilised Eppendorf tube, and left at room temperature for 5 min. Then 0.2 ml of chloroform was added and vortexed for 15 s, and left at room temperature for 15 min. The sample was then centrifuged at 12000 × *g* at 4 °C for 15 min. The upper aqueous phase was taken from the Eppendorf tube and 750 ml of 8 M LiCl was added into it followed by vortexing and incubation overnight at 4 °C. The sample was centrifuged for 15 min at 14000 × *g* and the pellet was resuspended in 500 ml isopropanol followed by ethanol precipitation. The pellet was dried briefly at room temperature and resuspended in 50 μl nuclease free water. The purity of total RNA was assessed spectrophotometrically by absorbance measurements at 260 and 280 nm.

2.5.2. cDNA synthesis

First-strand cDNA was synthesised in a reaction mixture containing 2 μg of total RNA as a template, 80 ng of random hexamer primers (Promega), 1× M-MLV buffer (Promega), 3 mM $MgCl_2$ (Promega), 10 mM dithiothreitol (Promega), 0.5 mM each dNTP (Promega), 40 U RNasin ribonuclease inhibitor (Promega), 200 U Moloney murine leukaemia virus reverse transcriptase (Promega), 40 U RNasin ribonuclease inhibitor (Promega) and 2 μg of bovine serum albumin in a 20 μl reaction volume and incubated at 37 °C for an hour. The reaction was terminated at 65 °C for 10 min. Synthesised cDNA was kept on ice, or stored at −20 °C if not used immediately.

2.5.3. Semiquantitative reverse transcription-polymerase chain reaction (sqRT-PCR)

Gene specific primers were based on conserved regions between previously described LME encoding gene sequences from *Trametes* species and closely related fungi. The sequences of these primers were as follows: *lcc* forward (5′-GATGGAGTCGACATTCAGG-3′) and reverse (5′-ATTGGCACGGCTTCTTCCA-3′), *mnp* forward (5′-TATAAGACCATGGCGGGGATGAG-3′) and reverse (5′-GATGAGCTCACCGGTGGTGATGC-3′), *lip* forward (5′-ACA AGG CAA GAC AGG CGA-3′) and reverse (5′-GACAAGATGTTGTGGTTC-3′). The amplified product sizes for *lcc*, *mnp* and *lip* are 578, 612 and 862 bp, respectively.

β-tubulin (*btub*), a constantly expressing house-keeping gene, was used as an internal control to normalise differences in the total RNA input or in the reverse transcription reaction efficiency. The sequences of *btub* primer are; forward (5′-TGGGCGAAGGGTCACTACAC-3′) and reverse (5′-GGGATCCACTCGACGAA-3′) (accession number AY131274). The amplified product size is 876 bp. The reaction mixture contained 2 μl cDNA aliquot, 1× Qiagen PCR Buffer (Qiagen, Valencia, CA, USA), 200 μM of each dNTP, 0.5 pmol of each primer and 2.5 U *Taq* DNA polymerase (Qiagen). Total reaction volumes were adjusted to 50 μl with sterilised water. Amplification was performed with a DNA thermal cycler Flexigene (Techne, Cambridge, UK) with a number of cycles of denaturation. An initial denaturation step at 94 °C for 3 min, followed by cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C for *lcc* and *lip*, at 58 °C for *mnp*, at 60 °C for *btub*), and extension (1 min at 72 °C), and one final extension step at 72 °C for 5 min were used. The samples were stored at −20 °C if not used immediately.

To validate the sqRT-PCR reactions, a series of RT-PCR reactions were sampled at different cycles and analysed by elec-

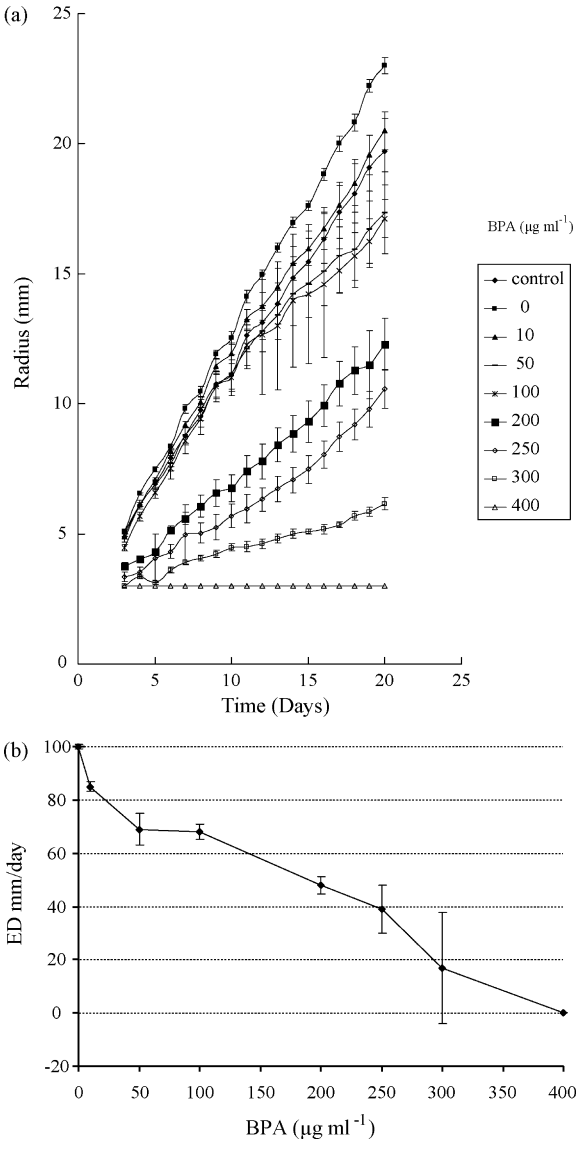

Fig. 1. The effect of BPA on fungal growth. BPA in DMF at 0–400 μg ml^{-1} was added separately to NL agar plates of *Trametes versicolor* and incubated for 20 days. The fungal diameters were measured daily to obtain the radius. Four replicates were prepared for all treatments. The temporal radius was plotted and the linear regression used to determine growth rate (mm day^{-1}) with S.E.M. indicated by error bars. (a) The colour lines indicate the final concentration dissolved in NL medium. Control is without solvent. For each point, values are presented as the mean ± S.E.M. from all replicates and (b) ED of *T. versicolor* by BPA. Error bars indicate the standard error of the ED estimated for each set of data except the control shown in (a). *Abbreviations*: bisphenol A (BPA) and effective dose (ED).

trophoresis to ensure that product abundance was evaluated in the exponential phase of the reaction. The number of cycles used in experiments was 32 for *lcc*, 27 for *mnp*, 30 for *lip* and *btub*. Fifteen microlitres of RT-PCR product was loaded onto a 2% agarose gel and electrophoresed. The gel image was then used for evaluation of the gene expression levels, normalised by *btub* with the ratio of (the means of each LME encoding gene/the mean of *btub*) using the Image J software (NIH, USA available from http://rsb.info.nih.gov/ij/). Statistical analysis was performed to compare the normalised expression levels between the control and each treatment using Statistica ver. 7 software (StatSoft Ltd., Bedford, UK). Differences were considered as significant at $P < 0.05$.

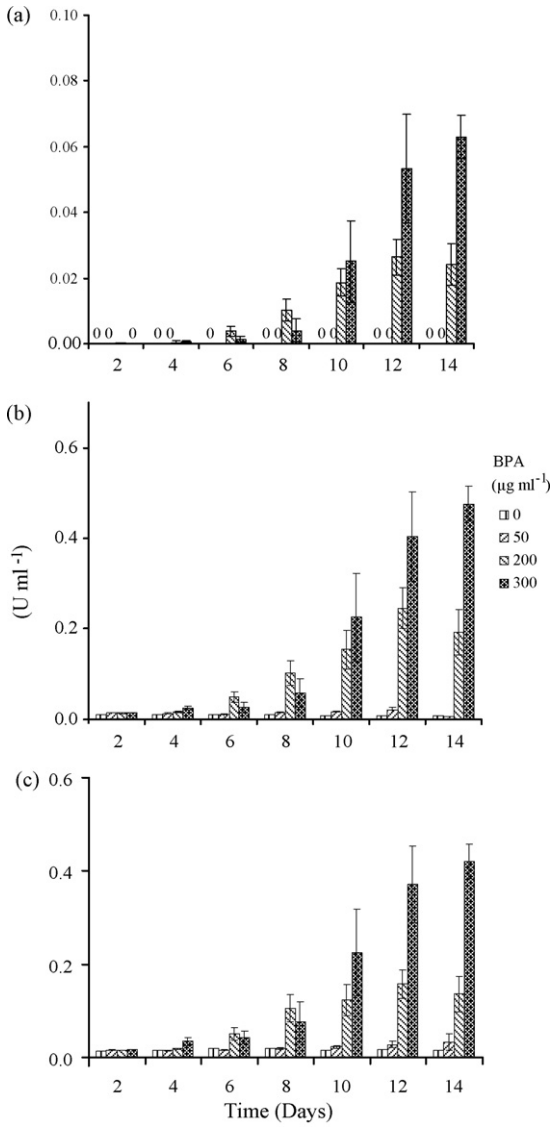

Fig. 2. LME production by *T. versicolor* grown with BPA. BPA in DMF at 0–300 μg ml^{-1} were added separately to the liquid culture of *T. versicolor* and incubated for 14 days. Culture fluid (2 ml) was taken from each treatment every 2 days and used to assay enzyme activity. Five replicates were prepared for each treatment. For each point, values are presented as the mean ± S.E.M. from all replicates. (a) LCC, (b) MNP and (c) LIP. The lines indicate the final concentration of BPA in medium (μg ml^{-1}). *Abbreviations*: bisphenol A (BPA), laccase (LCC), manganese peroxidase (MNP) and lignin peroxidase (LIP).

3. Results and discussion

3.1. Effect of BPA on fungal growth

The inhibitory effect of BPA on fungal growth was examined on NL plates supplied with various concentrations of BPA (Fig. 1a). Mycelial growth rate decreased as concentrations of BPA were increased. *T. versicolor* showed mycelial growth at high concentrations of BPA (up to 300 μg ml^{-1}) in nitrogen-limited conditions. The presence of DMF in the cultural medium (BPA, 0 μg ml^{-1}) stimulated fungal growth. From the radial growth rates, the approximate ED of BPA was estimated (Fig. 1b); $ED_{75} = 79.04$ μg ml^{-1}, $ED_{50} = 184.74$ μg ml^{-1} and $ED_{25} = 290.45$ μg ml^{-1}. Approximate ED doses (50, 200 and 300 μg ml^{-1}) were used in further enzyme activity assays and molecular studies.

3.2. Effect of BPA on LME activities

Fig. 2(a)–(c) shows the effect of BPA on quantities of LME produced by *T. versicolor*. These were all increased by BPA at 200 and 300 μg ml^{-1} ($P < 0.05$), despite an inhibition of fungal growth.

Higher enzyme activities at higher BPA concentrations possibly play a role in fungal survival under BPA-stress conditions.

3.3. Effect of BPA on LME encoding gene expression

Fig. 3 shows the effect of various concentrations of BPA on LME encoding gene expression. Total RNA was purified on

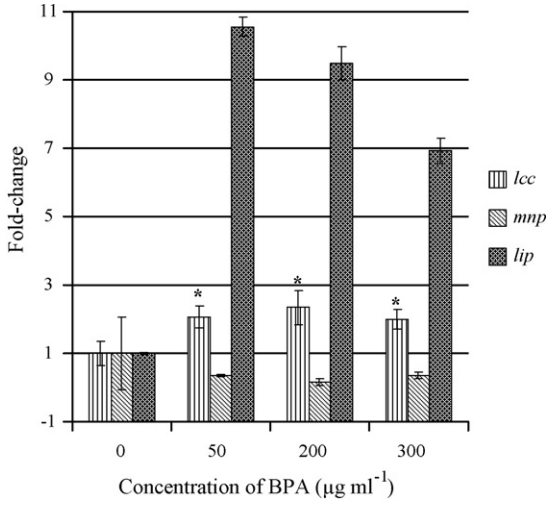

Fig. 3. The effect of various concentration of BPA on LME encoding gene expression. BPA in DMF at 0–300 μg ml^{-1} were added separately to the liquid culture of *T. versicolor* and incubated for 10 days. Total RNA isolated from each treatment was reverse transcribed and subjected to PCR. Triplicates were prepared for each treatment. A representative autoradiogram shows sqRT-PCR analysis of each LME encoding gene. The mean of each gene expression was normalised for *btub* and control expression within the same experiment. For each point, values are presented as the mean ± S.E.M. from all replicates. Bars marked with * are significantly ($P < 0.05$) different to the control. *Abbreviations*: bisphenol A (BPA), laccase (*lcc*), manganese peroxidase (*mnp*) and lignin peroxidase (*lip*).

day 10, just before LME activities reaching their maximum in Fig. 2. In a nitrogen-limited environment, *mnp* gene showed the highest expression level without BPA in *T. versicolor*; about 4-fold of *lcc* and 45-fold of *lip*. BPA decreased *mnp* transcript level; 0.16-fold at 200 $\mu g\,ml^{-1}$. The expression level of *mnp* was higher at 300 $\mu g\,ml^{-1}$ of BPA than 200 $\mu g\,ml^{-1}$. The *lcc* expression was significantly increased by BPA (at maximum, 2.3-fold by 200 $\mu g\,ml^{-1}$ of BPA ($P < 0.05$)). The *lip* expression was increased at all concentrations. The peak of induction level of *lip* expression was at the lowest concentration used (50 $\mu g\,ml^{-1}$), at this concentration its expression level was more than 10-fold higher than the control. The increased *lip* and *lcc* transcript levels may compensate for the *mnp* transcript level in the presence of BPA. This hypothesis may be explained by the fact that the three LMEs are encoded by the same gene families which allows complex regulation and production of multiple isoforms of LMEs [16]. Without BPA, no *lip* transcripts were observed. However, the expression of *lip* was turned on and strongly induced by the chemical stress of BPA, which may show the sensitivity of *lip* gene to the impact of BPA.

4. Conclusions

In summary, *T. versicolor* is resistant in terms of its fungal growth to chemical stress by BPA in culture in nitrogen-limited conditions. The LME activities had been enhanced by a high concentration of BPA. Transcript levels of the genes encoding non-specific LMEs in *T. versicolor* were altered in the presence of BPA. In particular, the LIP-encoding gene may be sensitive to the impact of BPA. LMEs and their encoding genes in this white-rot fungus are potentially sensitive monitoring factors for the impact of BPA. Exploitation of the fungus used in this study could be taken further by being used as a potent biologically based elimination system in BPA-contaminated sites.

Acknowledgement

We are grateful to the National Institute of Technology and Evaluation in Japan for data providing and financial support.

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