Biodegradation of pyrene during submerged cultivation of *Trametes versicolor*

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Abstract. Pyrene is one of the most persistent pollutants belonging to the group of high molecular weight polycyclic aromatic hydrocarbons. Due to the presence of fused benzene rings these compounds are extremely difficult for biodegradation. The white-rot fungi possess remarkably high potential when it comes to the biodegradation of toxic organic substances with aromatic rings due to their unique lignin-degrading enzymatic complex. In the present study, pyrene with concentrations up to 200 ppm was added to the nutrient medium during submerged cultivation of the higher basidiomycete mushroom *Trametes versicolor* NBIMCC 8939. The experiment continued for 20 days and samples were taken every 5 days. Analysis of the activities of the enzymes laccase and manganese-dependent peroxidase were performed as well as determination of the residual pyrene concertation and identification of the by-products of the degradation process. It was established that the highest pyrene removal was achieved when the lowest initial concentration was introduced to the medium. The strain *T. versicolor* NBIMCC 8939 was able to biodegrade 12% of the initially introduced 50 ppm pyrene in the medium.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic pollutants that are widespread in the environment. Their chemical structure includes at least two fused benzene rings usually containing carbon and hydrogen atoms, but there are functional derivatives such as nitro-PAHs and heterocyclic analogs like aza-arenes [1]. The sources of these compounds are multiple and with regard to their origin, they could be divided as diagenetic, pyrogenic, or petrogenic. When PAHs occur as a result of volcanic eruptions or degradation of organic matter they are classified as diagenetic. Pyrogenic PAHs occur in the environment as a result of incomplete combustion of organic matter and when the contamination is due to oil spills, petroleum processing, or other anthropogenic or natural causes related to petroleum the PAHs are petrogenic in origin [2]. Given this information, it is not difficult to conclude that their entrance into the environment is continuous. In the last 30 years, the scientific interest in PAHs has increased mainly regarding to the research on exposure, toxicity, and bioremediation of contaminated areas. Over 100 PAHs have been identified in environmental samples and over 200 in tobacco smoke [1,3]. These chemical compounds are soluble in many organic pollutants and are highly lipophilic, but have low water solubility. Due to their widespread in the environment, it is impossible for the human population to avoid exposure to PAHs. These compounds are reported as highly cancerogenic, possessing potential developmental and reproductive disrupting properties [4,5], and also neurotoxic, immunotoxic, and cytotoxic effects [2,6,7].

Due to their structure PAHs are considered as hard for degradation. Efforts have been made for the implementation of conventional degradation techniques but issues like high cost and waste disposal led to their failure. The most serious problem with these techniques is that usually, the intermediates resulting from the degradation are even more toxic than the degraded compound [8]. Thus, led to the search for an eco-friendly and economically beneficial technique based on the introduction of biological agents. Many microbial strains like bacteria, algae, and yeasts were objects of this research area, but recently fungi dragged the attention of the researchers because of their ability to degrade wood and lignin in particular, due to the presence of the unique lignin-modifying enzymatic system. The main enzymes in that system are laccase and peroxidases (lignin peroxidase, manganese-dependent peroxidase, manganese-independent peroxidase, and versatile peroxidase) but laccase, in combination with peroxidases, is the enzyme that is most commonly involved in the degradation of phenolic and non-phenolic compounds [9].

The strain used in the current study, *Trametes versicolor* NBIMCC 8939, is known for its ability to degrade industrial dyes, endocrine disrupting chemicals (like bisphenol A), phenolic compounds, PAHs [10-13] due to the enzymes present in the lignin-modifying complex of the fungus, namely laccase and manganese-dependant peroxidase (MnP). The strain was cultivated in submerged conditions and pyrene was introduced in different concentrations in the nutrient medium.

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2 Materials and methods

2.1. Fungal strain and maintains

The macromycete *T. versicolor* NBIMCC 8939 is a part of the mushroom collection of the Department of Biotechnology at the University of Food Technologies in Plovdiv, Bulgaria. The strain was previously isolated, identified, and deposited at the National Bank of Industrial Microorganism and Cell Cultures. Czapek-Dox agar (Table 1) with pH 6.5 was used for the growth and maintenance of the strain.

Table 1. Czapek-Dox agar composition.

Component	Concentration, g/L
Sucrose	30.0
Yeast extract	5.0
$NaNO_3$	2.0
K_2HPO_4	1.0
$MgSO_4$	0.5
KCl	0.5
$FeSO_4$	0.01
Agar	15

The culture was developed on slant Czapek-Dox agar for 7 days and stored at 4°C afterward.

2.2. Culture conditions and biodegradation

For inoculum obtaining a 7-day-old culture of *T. versicolor* was used for the preparation of a suspension of vegetative cells. Liquid Czapek-Dox medium (100 mL) in a 500 mL flask was used for the development of the vegetative inoculum which was applied in the biodegradation process. Three types of media, based on Czapek-Dox medium were used for the assessment of the biodegradation potential of the strain (Table 2): Czapek-Dox in its full composition (CDF); Czapek-Dox with low sucrose concentration (CDL) and Czapek-Dox with no sucrose (CDN).

Table 2. Biodegradation media composition.

Component	CDF, g/L	CDL, g/L	CDN, g/L
Sucrose	30.0	10.0	-
Yeast extract	5.0	5.0	5.0
$NaNO_3$	2.0	2.0	2.0
K_2HPO_4	1.0	1.0	1.0
$MgSO_4$	0.5	0.5	0.5
KCl	0.5	0.5	0.5
$FeSO_4$	0.01	0.01	0.01

pH of each medium was set to 6.5 and sterilization was carried out at 118°C for 30 min. After cooling, pyrene in increasing concentrations up to 200 ppm, was introduced to the media. The control samples for each media were without pyrene. The flasks were then inoculated with 5% w/v pellets from *T. versicolor* and placed on an orbital shaker at 220 rpm and 28°C for 20 days.

2.3. Determination of biomass concentration

The concentration of the biomass was determined by measurement of the absolute dry weight after separation of the biomass from the cultural broth. The separation itself was performed by filtration and afterward, the biomass was dried to constant weight at 105°C using a RADWAG moisture analyzer (RADWAG, Poland) and was expressed as g DW/L.

2.4. Determination of enzyme activities

The activity of laccase was determined according to the method of Ride, using syringaldazyne as substrate [14]. The reaction mixture with a final volume of 300 µL consisting of 220 µL 50 mM phosphate buffer (pH4.5) and 50 µL cultural broth was placed in the chamber of a SpectroStar Nano (BMG Labtech, Germany) spectrophotometer at 37°C for 2 min. The substrate (0.216 mM syringaldazyne solution in methanol) was added to the mixture and the changes in the absorbance values were monitored for 5 min. One unit of laccase activity corresponds to a 0.001 change in the absorbance at the reaction conditions and is expressed as U/mL.

The oxidation of Mn (II) to Mn (III) at 270 nm was used as an indicator for the activity of manganese-dependent peroxidase [15]. Solution of $MnSO_4$ (1mM) was prepared in 50mM sodium-malonate buffer with pH 4.5. The reaction mixture contained 140 µL of the solution and 20 µL cultural broth. The oxidation was initiated with the addition of 40 µL 1mM H_2O_2 and the absorbance at 270 nm was recorded after 10 min. One unit of MnP activity is defined as the quantity of enzyme required to oxidize 1 µmol $MnSO_4$ for 1 minute at the reaction conditions.

2.5. Extraction of pyrene

Residual pyrene was extracted from the cultural broth with hexane. The following extraction procedure was applied. Samples were mixed with hexane in a 1:1 ratio and placed on an orbital shaker at 150 rpm for 1 h. The sample was then transferred in a separation funnel, mixed vigorously for 90 s, and left for full separation of the phases. The organic fraction was collected and the sample was put through two additional extraction procedures with hexane with a ratio of 1:0.5 in favor of the sample. The organic fractions of the three extractions were mixed and anhydrous $NaSO_4$ was added for elimination of the water. Samples were then evaporated under vacuum until dry, resuspended in 5 mL acetonitrile (HPLC grade), and used for chromatographic analysis.

2.6. Chromatographic analysis

High-performance liquid chromatography was used for the determination of the residual concentration of pyrene in the samples. The analysis was performed on Agilent 1200 Infinity Series (Agilent Technologies Inc., USA) equipped with a UV detection module and Zorbax Eclipse PAH separation column (5µm; 4.6nm x 150 mm). The mobile phase was 100% acetonitrile (HPLC grade) at a flow rate of 1 mL/min and the detection was made at 220 nm. Samples (20 µL) were analyzed in triplicate and the mean was calculated.

The intermediate products of the biodegradation of pyrene were determined on GC Trace 3000 with TR-5MS (30m × 0.25mm ID × 0.25µm) column equipped with single quadrupole MS ISQ QD (Thermo Fisher Scientific Inc., USA). The 1 µL sample was injected in split mode. The temperatures of the oven were as follows: 3 min at 60°C, increasing with 15°C/min up to 310°C, and holding for 5 min. The temperature of the transfer line was 260°C and 220°C for the ion source. Helium was used as mobile phase at a flow rate of 1 mL/min, ionization at 70 eV, m/z 35-350.

2.7. Statistical analysis

All experiments were performed in triplicate and the values are expressed as mean ±SD. Statistical significance was evaluated by analysis of variance (ANOVA, Tukey`s test; values of $p<0.05$ indicated statistical significance).

3 Results and discussion

Pyrene is considered difficult for biodegradation because of the specificity of its structure and the presence of four fused benzene rings. However, many scientific reports describe the ability of microorganisms to degrade pyrene with a focus on the fungal representatives. The white-rot fungi (*Basidiomycota*) pose a unique lignin-degrading enzymatic complex, which can break down complicated structures, such as extremely resistant to microbial attacks lignin, bisphenols, nonylphenols, synthetic dyes, etc. [10, 13, 16, 17]. Different basidiomycete species are often used as model organisms for implementation in degradation processes. The microbial agent used in the current study *T. versicolor* NBIMCC 8939, can fully degrade phenolic compounds and PAHs and also to produce laccase and manganese-dependent peroxidase with high activities [10-13, 18].

Three types of media (Table 2) were used for the implementation of the biodegradation process. After the inoculation biomass gain was monitored during the whole process. When the biodegradation process was made in CDN medium there was no fungal growth detected during the whole experiment. This could be explained by the absence of a carbon source in the medium. When CDL was used growth was observed in the control sample, reaching a concentration of 8 g DW/L on the 10th day. In the flasks containing 50 ppm and 100 ppm pyrene the growth was negligible (7 and 6 g DW/L on the 10th day, respectively) and when higher concentrations were applied there was no growth detected. The strain was able to grow only when CDF was used as a medium (Figure 1). In the control sample, the biomass concentration reached 17 g DW/L in 10 days, but with the introduction of pyrene in the medium there was inhibition of the growth. When 50 ppm of pyrene was present in the medium the maximum biomass concentration was 13 g/L on the 10th day. In a medium with 100 ppm pyrene, the maximal biomass gain was 11 g DW/L and was lowered with the increase of pyrene concentration – 10 g DW/L and 8 g DW/L for 150 ppm and 200 ppm respectively.

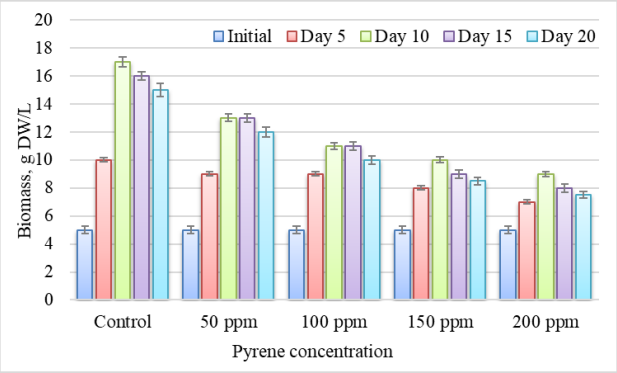

Figure 1. Biomass concentration of *T. versicolor* in CDF with pyrene in submerged culture

The activities of laccase and MnP were monitored during the degradation process of pyrene and the data is presented in Figure 2.

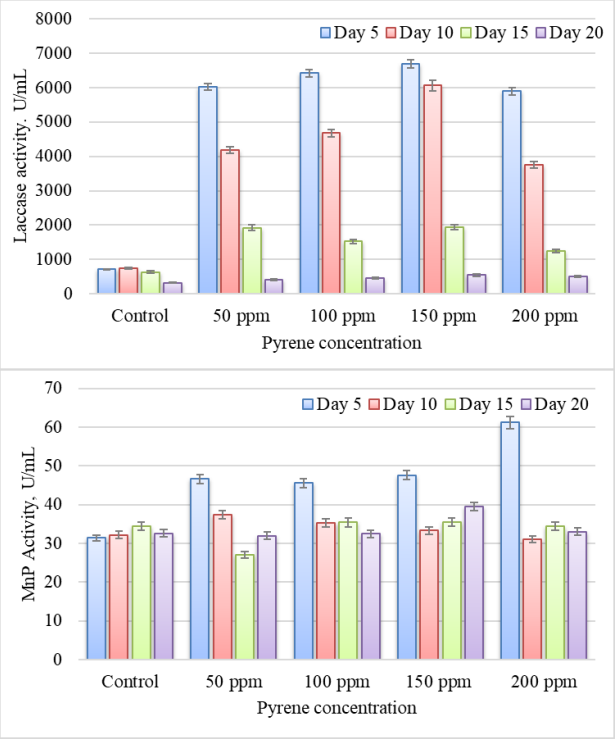

Figure 2. Activity of laccase (A) and MnP (B) during pyrene biodegradation by *T. versicolor* in CDF

It is clear that the presence of pyrene in the degradation medium leads to the induction of the activity of laccase.

The highest activity was increased nearly 5-fold compared to the control on the fifth day of the experiment in all samples as shown in Figure 2A. It is not unusual for compounds containing aromatic rings to induce the laccase activity. There are reports regarding the possibility of induction of laccase from *T. versicolor* by the application of various aromatic compounds, such as ferulic acid, 2,5-xylidine, veratryl alcohol, etc. [21, 22]. Xenobiotics, such as Bisphenol A also demonstrate inductive properties towards the laccase synthesis by *T. versicolor* [13]. It has been reported that in the presence of pyrene with an initial concentration of 50 mg/L, during submerged cultivation of *Pleurotus ostreatus* the activity of the enzyme increased from 11.7 U/mL in the control to 26.8 U/mL on the fourth day. Laccase activity was also induced in the presence of other PAHs, such as anthracene, fluorene, chrysene, and others [23]. Similar results were observed regarding the activity of MnP (Figure 2B), but in this case, the induction is not so significant. The maximal activity was observed on the fifth day of the experiment, reaching a value of 61 U/mL in the samples containing 200 ppm pyrene or nearly 2-fold escalation compared to the control sample without pyrene. Pozdniakova et al. [23] reported that in the presence of pyrene, the peroxidase activity was increased by nearly 13% during submerged cultivation of *P. ostreatus* [23], whereas in our study the activity of MnP was increased 2-fold.

The degradation rate of pyrene during submerged cultivation of *T. versicolor* in CDF medium is presented in Figure 3. The highest biodegradation of pyrene was measured on the 20th day of the experiment, where the concentration of the residual pyrene in the sample was 44 ppm (in the sample with 50 ppm initial concentration). Increasing the concentration of the compound led to lower degradation values.

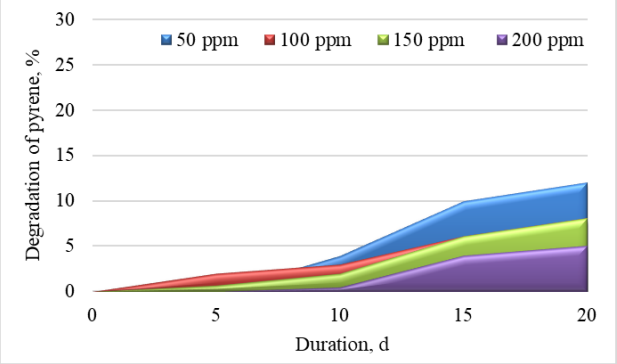

Figure 3. Biodegradation of pyrene by *T. versicolor* in CDF

Despite the high activities of the enzymes, the level of degradation of pyrene in these experimental conditions is quite low and reaches 12% in the samples with an initial concentration of pyrene 50 ppm. Laccase and MnP are key enzymes in the degradation of xenobiotics, and the induction of their activities in the presence of PAHs is not always related to better degradation. Ting et al. studied the possibilities for biodegradation of pyrene by *Ganoderma lucidum*, where the four different strains were able to degrade between 11% and 19% pyrene with an initial concentration of 20 mg/L [24], thus showing that the degradation ability could be related to specific characteristics of the strain's metabolism. Studies are proving that the introduction of other types of inductors in the medium, such as citric acid, gallic acid, or $CuSO_4$ could enhance the degradation of phenanthrene and pyrene in submerged culture [24]. Other studies suggest that the supplementation of the culture with Mn^{2+} could lead to higher activities of the MnP enzyme and therefore higher degradation rates of PAHs [25, 26].

Many compounds were identified after the analysis of the results obtained by the GC-MS, but only a few could be related to pyrene degradation pathways presented in the literature (Figure 4). Even at their low degradation rates, the presence of dibutyl phthalate (Figure 4A); α-ethyl benzeneacetaldehyde (Figure 4B), and Phenaleno[1,9-bc]thiophene (Figure 4C) proves that pyrene is been an object of biodegradation under aerobic conditions.

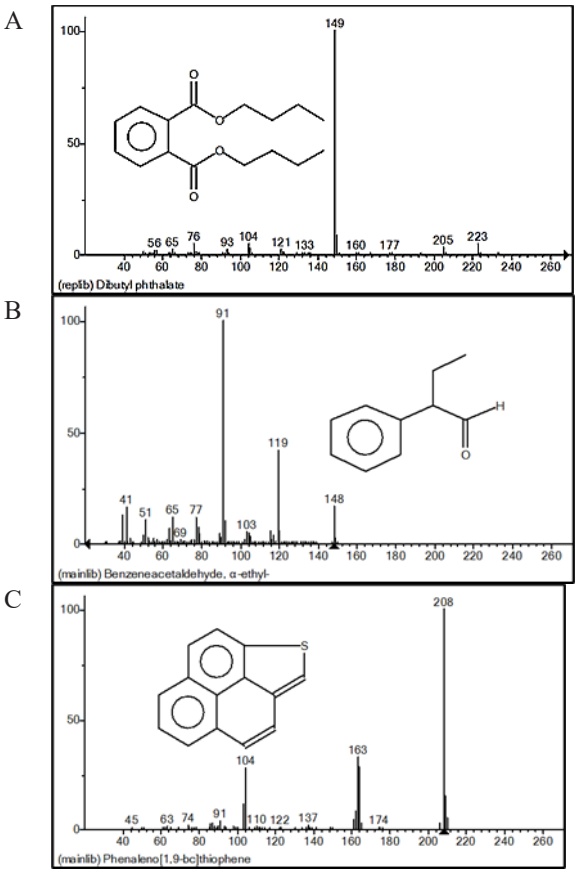

Figure 4. Identified pyrene degradation products: Dibutyl phthalate (A); α-ethyl benzeneacetaldehyde (B) and Phenaleno [1,9-bc] thiophene (C)

The aerobic degradation of pyrene was described by Agrawal et al. [27]. Laccase oxidized pyrene to pyrene oxide, followed by conversion to trans 4,5-pyrene dihydrodiol. The latest was then dehydrogenated to 4,5-dihydroxypyrene which was oxidated by the enzymes laccase, lignin peroxidase, and MnP resulting in ring cleavage and obtaining of phenanthrene and esters of the phthalic acid. With the action of the phenol oxidases and

peroxidases, benzoic acid was formed and converted afterward to pyruvic acid, which was then metabolized to carbon dioxide.

The identified products could be considered as derivatives of the compounds described above and most likely are intermediates of the aerobic pyrene degradation. The degradation process could be enhanced by a variation of parameters such as temperature, agitation, illumination, pH, etc. Also, the medium composition and the initial pyrene concentration could significantly affect the efficiency of the process. The introduction of compounds serving as mediators for the laccase activity in the medium also could intensify the degradation process. Having all this in mind it is possible higher pyrene degradation rates to be obtained with *T. versicolor* NBIMCC 8939 under submerged cultivation conditions. Further studies are needed for optimization of the parameters of the process thus achieving higher degradation rates.

4 Conclusion

The molecule of pyrene consists of four fused benzene rings, which increases its endurance to microbial degradation. On the other hand, the enzymatic ligninolytic complex of the white-rot fungi allows them to degrade persistent pollutants such as PAHs. *T. versicolor* NBIMCC 8939 demonstrated the ability to synthesize high activities of the enzymes laccase and MnP under submerged conditions in the presence of pyrene. The strain was able to degrade 12% of pyrene with an initial concentration of 50 ppm. The identified intermediates were associated with degradation products of the aerobic degradation pathway of the compound. Further experiments are needed for the establishment of optimal degradation conditions and achieving higher degradation rates.

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