Bulgarian Chemical Communications, Volume 56, Special Issue D2 (pp.78-83) 2024 DOI: 10.34049/bcc.56.D.S2P46

Degradation of pyrene by laccase from *Trametes versicolor*

M. S. Brazkova[1*], R. Y. Koleva[2], G. V. Angelova[1], A. I. Krastanov[1]

[1]*Department of Biotechnology, University of Food Technologies, 26 Maritza Blvd., Plovdiv, Bulgaria*
[2] *Department of Chemical Technologies, Burgas 'Asen Zlatarov' University, 1 Prof. Yakim Yakimov Blvd., Burgas, Bulgaria*

Received: November 3, 2023; Revised: April 11, 2024

In recent years the development of eco-friendly remediation technologies with economical advantage is based on the incorporation of microorganisms or their enzyme systems in the degradation processes. In the present study purified laccase with specific activity 105.8 U/mg, obtained after submerged cultivation of the basidiomycete *Trametes versicolor,* was used in free and immobilized form for pyrene degradation. The alginate gel entrapment method and the encapsulation in amphiphilic dendritic-linear-dendritic block copolymers were applied as immobilization techniques. The free form of the enzyme was able to reduce the concentration of pyrene by 15 ppm to a final concentration of 185 ppm at the 20th day. The application of the enzyme, encapsulated in amphiphilic dendritic-linear-dendritic block copolymers, resulted in a decrease of the concentration of pyrene ranging from 4 ppm to 28 ppm for the different preparations. The degradation of pyrene with laccase immobilized in Ca-alginate gel led to a decrease in the concentration of the compound by 26 ppm for 20 days.

Keywords: Laccase, pyrene, immobilization, encapsulation, amphiphilic polymers

INTRODUCTION

The processes of vast industrialization and urbanization in recent years are tightly connected with the increasing concentrations of xenobiotic compounds in the environment. These compounds are used as intermediates in various industrial processes including the production of pesticides, personal care products, disinfectants, polycyclic aromatic hydrocarbons (PAHs), pharmaceutically active compounds, phenolic compounds and other chemicals [1]. Because of their high toxicity, they could be related to many health conditions, such as infections of the respiratory tract, disruption of the endocrine system, development disorders, and carcinogenic and mutagenic effects [2, 3]. On the other hand, they can cause harmful effects towards the ecosystems as well, due to their ability to accumulate in the food chain.

Polycyclic aromatic hydrocarbons are a group of over a hundred organic compounds widely spread in the environment. The level of their toxicity is strongly related to their structure and mostly to the number of fused benzene rings in their molecules. The persistence of these compounds makes them a serious threat to animal and human health and also leads to environmental pollution. Pyrene is a polycyclic aromatic hydrocarbon which has 4 fused benzene rings in its molecule thus making it extremely resistant to microbial degradation. It is one of the model compounds used in PAHs degradation studies because it is abundantly found in the contaminated environment [4]. This compound enters the environment mostly through the combustion of diesel fuel, but it also is a result of the production of bleaching agents and dyes. Pyrene and its derivatives have many applications due to their unique properties such as blue emission, high chemical stability and charge-carrier mobility in diverse scientific fields like organic light-emitting diodes (OLEDs), organic field-effect transistors (OFETs) and organic photovoltaic cells (OPVs) [5]. Having in mind these factors it is easy to suppose that xenobiotics represent serious environmental problems and the urge to develop a successful detoxification technique is exigent.

Many conventional techniques used for the treatment of xenobiotic contamination failed in the research process mainly because of problems like waste disposal and high costs, but mostly because the obtained degradation products are in some cases even more toxic than the initial compound. This hazardous stage is supposed to be avoided by the implementation of biological agents in the degradation processes leading to economically advantageous and eco-friendly technology. Several bacterial representatives have proven their ability to use pyrene as a sole source of carbon and energy

* To whom all correspondence should be sent:
E-mail: *mbrazkova@uft-plovdiv.bg*

but this capability is presented only in laboratory-scale studies. Some white-rot fungi (WRF), macromycetes and ectomycorrhizal fungi amongst all species in the fungal kingdom are also able to fully mineralize PAHs [6]. *Trametes versicolor*, a WRF, is known for its ability to degrade various aromatic compounds, due to the synthesis of a unique enzymatic complex, containing ligninases, oxidases and laccases [7–10]. The enzymes of the lignin-degrading enzymatic system could be applied in the degradation process in native or purified form. The application of immobilized microbial enzymes in the degradation of PAHs is one of the prospective approaches because the immobilized enzymes can stay stable longer in unfavorable conditions in terms of temperature and pH. Also, these biocatalysts can be easily separated from the reaction mixtures and reused multiple times. Immobilized preparations obtained by entrapment, encapsulation, adsorption and covalent binding have been studied for their degradation capacity towards PAHs [11]. On the other hand, laccases have broad substrate specificity but only for water-soluble substrates. By the encapsulation of the enzyme in dendritic-linear-dendritic (DLD) block copolymers its substrate specificity could be extended because these polymers can selectively adhere to various surfaces and bound hydrophobic substances in aqueous medium.

The aim of this study is the evaluation of the effectiveness of purified free and immobilized laccase from *Trametes versicolor* to degrade pyrene in an aqueous medium.

MATERIALS AND METHODS

All reagents used in the experiments were of analytical grade unless otherwise stated. Pyrene (CAS Number 129-00 0,98%), acetonitrile (HPLC grade), sodium alginate and n-hexane were purchased from Merck KGaA (Germany). The DLD block copolymers were generously provided by the Department of Chemistry of SUNY College of Environmental Science & Forestry, Syracuse, NY, USA.

Microorganism and cultivation conditions

The basidiomycete *Trametes versicolor* NBIMCC 8939 is maintained on Chapek-Dox agar medium with the following composition (g/L): sucrose – 30.0; yeast extract – 5.0; $NaNO_3$ – 2.0; K_2HPO_4 – 1.0; $MgSO_4.7H_2O$ – 0.5; KCl -0.5; $FeSO_4.7H_2O$ – 0.01 and agar – 15.0 with pH 6.5 and stored in tubes at 4°C.

Liquid Chapek-Dox medium with the addition of 6mM 2-methoxyphenol was used for the enzyme synthesis. After 144 h of cultivation on a rotary shaker at 220 rpm and 28°C the cultural broth was separated from the biomass by centrifugation at 6000 rpm for 10 minutes and the protein content and laccase activity were determined in the broth, as well as the concentration of dry biomass.

Enzyme activity and protein determination

Syringaldazyne was used as a substrate for the evaluation of the laccase activity where the change of the absorption at 530 nm was monitored for 5 minutes at 37°C. The reaction mixture with a total volume of 300 μL consists of 220 μL potassium-phosphate buffer (50 mM, pH 4.5), 30 μL substrate solution (0.216 mM in methanol) and enzyme solution. One unit of enzyme activity corresponds to 0.001 change in OD at the reaction conditions and is expressed as units per mL. The total protein concentration was evaluated according to the "Bradford" method [12].

Isolation and purification of laccase

After the cultural broth was collected laccase was isolated as previously reported [7]. Briefly, the cultural broth was separated from the biomass by filtration through a glass-fibre filter followed by ultrafiltration through a 30 000 Da membrane on Millipore Prep/Scale-TFF unit and 400 mL pressure cell. After size-exclusion chromatography on Sephacryl 200-HR the blue fractions were collected and concentrated again for obtaining of a homogeneous enzyme preparation.

Immobilization

The obtained enzyme was immobilized by two methods – by gel entrapment and by encapsulation. For the gel entrapment method, a 2% sodium alginate solution was prepared with constant stirring and heating. The obtained alginate solution was cooled and the enzyme in a final concentration of 2 mg/mL was added and mixed well. The mixture was added dropwise with a syringe and a G21 needle into cooled (4°C) 0.5% solution of $CaCl_2$ with constant gentle stirring which continued for 1 h after the last drop was introduced to the solution. Afterwards, the formed Ca-alginate beads were separated from the solution through filtration and washed twice with distilled water. The loading efficiency and immobilization effectiveness were calculated according to Daasi *et al*. [13].

For the encapsulation of the enzyme dendritic-linear-dendritic copolymers were used. Those polymers were synthesized from reactive poly (benzyl ether)monodendrons of the second [G-2], third [G-3] and fourth [G-4] generation by coupling

with poly(ethylene glycol)s with a molecular weight of 5 kDa or 10.8 kDa [14]. Laccase aqueous solution with a concentration of 2mg/mL was mixed with 0.007 g of each DLD polymer in a final volume of 10 mL. The mixtures were placed on a rotary shaker for 12 h at 4 ℃ followed by 8 h at room temperature [7].

Pyrene removal by free and immobilized laccase

An aqueous solution of purified laccase with a final concentration of 2 mg/mL was used as a medium for the degradation of pyrene. The PAH was added to the aqueous mixture to reach a final concentration of 200 ppm. The control samples contained 200 ppm pyrene in aqueous medium. The experimental tubes were placed on a rotary mixer at 25°C in absence of light for 20 days. Samples were taken daily for spectral and HPLC analysis.

Deionized water was used for the degradation of pyrene by the Ca-alginate laccase preparation. Each test tube contained 200 ppm pyrene and 1 g immobilized preparation. The control samples contained 200 ppm pyrene and 1 g Ca-alginate beads without enzyme. The tubes were placed on a rotary shaker at 25°C in darkness for 20 days and samples were taken daily for further examinations.

Pyrene in a final concentration of 200 ppm was added to the enzyme-copolymer complex and the experimental tubes were placed on a rotary shaker at 25°C in the absence of light. The control samples contained DLD polymers in aqueous medium and 200 ppm pyrene. Samples were taken daily for spectral and HPLC analysis.

Spectral analysis

The spectral analysis was conducted on UV-VIS spectrophotometer SpectroStar NANO (BMG Labtech, Germany). Samples were taken daily until the 20th day of the experiment. 200 µL aliquot of each test tube was placed in a 96-well UV plate and the spectrum was recorded in the range of 220-400 nm with a resolution of 2 nm.

Extraction of pyrene

The samples were extracted threefold with hexane for the determination of the residual pyrene concentration. Anhydrous Na_2SO_4 was then added to the collected fractions to remove residual water from the samples. Afterwards, they were transferred into a flask with appropriate volume and evaporated under vacuum until completely dry. Acetonitrile (5 mL) was used for dissolution of the dry residue. The samples were then filtered through a syringe filter (0.45 µm) and used for determination of pyrene concentration.

HPLC analysis

Residual pyrene concentration was determined on Agilent Infinity 1220 HPLC system equipped with UV-VIS detector and Agilent Zorbax Eclipse PAH (5 µm, 4.6×150 mm) column. Acetonitrile was used as mobile phase with a flow of 1 mL/min, injection volume was 20 µL, and detection was made at 220 nm wavelength. The retention time for pyrene at these conditions was 2,73 min.

RESULTS AND DISCUSSION

The evaluation of the ability of laccase to degrade pyrene in aqueous medium was made by the comparison of the data obtained after implementation of free enzyme, enzyme entrapped in Ca-alginate gel and enzyme encapsulated in DLD polymers. To our knowledge to date, there is no other study exploring the degradation of pyrene in aqueous medium by immobilized laccase entrapped in amphiphilic polymers.

As a result of the cultivation process, cultural broth with laccase activity of 6545 U/mL was obtained. After the purification procedures, the enzyme preparation had a specific activity of 105.8 U/mg protein and a protein content of 56 mg/mL. After appropriate dosage, the enzyme was used in free form or immobilized form for the degradation of pyrene.

A summary of the data obtained during the spectral analysis is presented in Fig. 1. The differences between the different samples are visible. When the free purified enzyme was introduced to the reaction mixture there were almost no visible changes in the spectra during the whole experiment (Fig. 1A). On the other hand, the sample with the entrapped in Ca-alginate laccase demonstrated significant changes in the spectra (Fig. 1B). Samples taken on the 10^{th}, 15^{th} and 20^{th} day show lower absorbance values in the monitored range. These changes in the spectra must be due to oxidation processes of pyrene during the experiment. The formed new intermediates are the reason for the decrease in the absorbance values. Many studies discuss the ability of bacteria immobilized in alginate [15] and fungal mycelium [16] to degrade PAHs, but the information regarding the sole application of laccase in such process is very scarce. Fig. 1C presents the spectral data obtained with laccase encapsulated in [G-2] polymer.

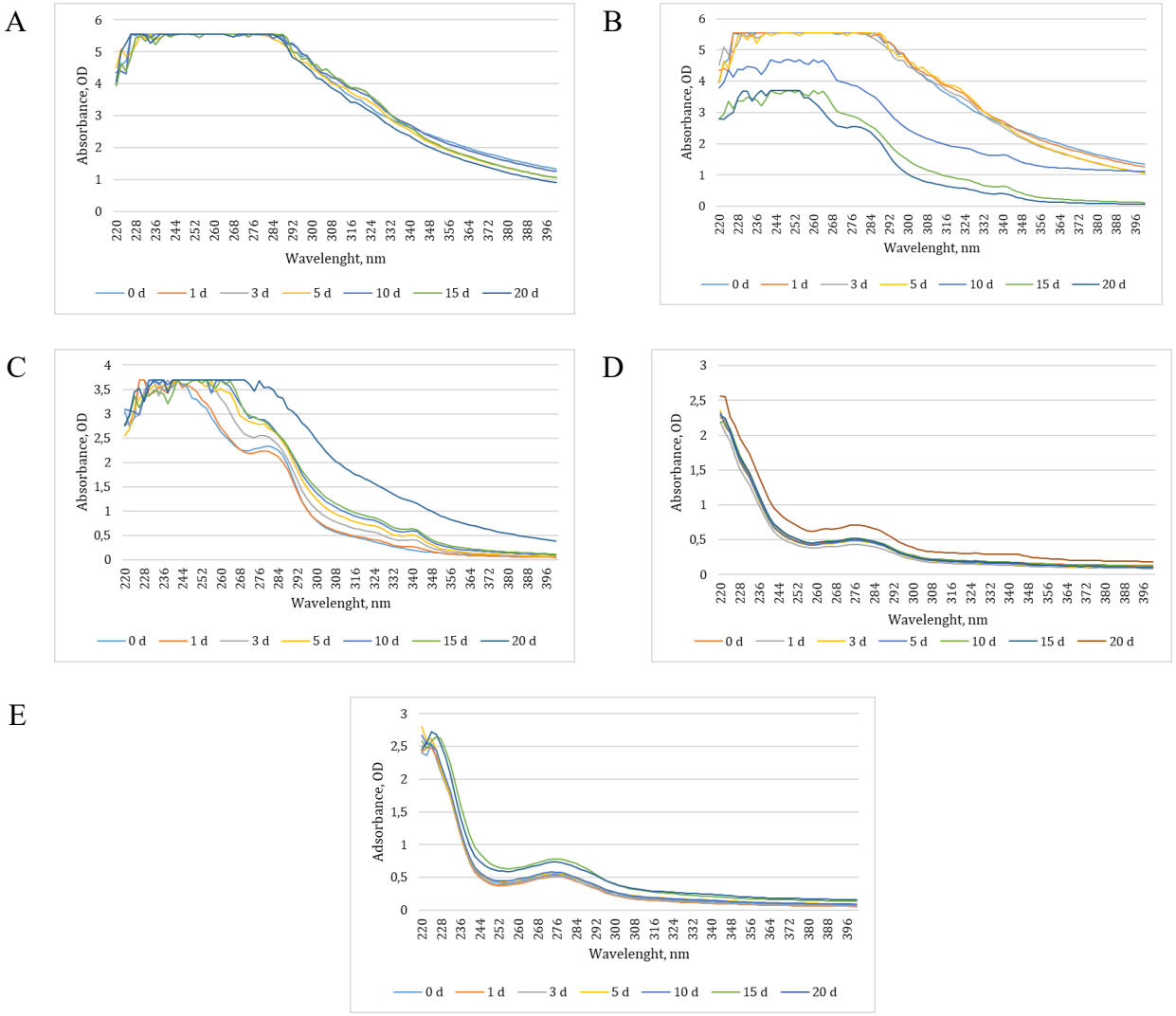

Fig. 1. Spectral data of the reaction mixture between A) Purified laccase and pyrene; B) Entrapped laccase and pyrene; C) Laccase, modified with [G-2] block copolymer and pyrene; D) Laccase, modified with [G-3] block copolymer and pyrene; E) Laccase, modified with [G-4] block copolymer and pyrene.

The changes in the graph are visible, but yet not as intensive as the ones in Fig. 1B. A slight increase of the absorbance values was recorded during the experiment and the highest values were observed on the 20th day. With laccase encapsulated in [G-3] and [G-4] polymers, the spectral changes occurred around the 15th day of the experiment and represented a minor increase in the spectral curves, most likely because of the oxidation processes that took place in the degradation system. It is important to state that when encapsulated in these polymers the enzyme is located in the central part, whilst the polymer envelops it, creating a hydrophobic surrounding. The monomers in the DLD polymers are different which explains the differences in the spectral data, and proves that in fact the polymer is based on poly(benzyl ether)s monodendrons of second generation and poly(ethylene glycol) with molecular weight 5000 Da ([G-2]). Since the water solubility of PAHs is very low it could be assumed that the polymer acts as a mediator for the pyrene oxidation resulting in higher transformation rates. The usage of amphiphilic block copolymers for laccase encapsulation is a relatively new but promising approach for the removal of persistent organic pollutants [7, 17]. Yet there is no other study in the available literature discussing their effectivity regarding pyrene transformation.

The changes in the spectra of the samples correspond to the data obtained by the quantitative analysis. Figure 2 shows the residual pyrene concentration after 20 days of incubation with the used laccase preparations. When free purified enzyme was applied to the reaction mixture for 20 days the pyrene concentration was by 15 ppm lower than the initial. The application of entrapped in Ca-alginate gel led to higher degradation values, reaching 174 ppm residual pyrene on the 20th day. The application of the enzyme encapsulated in DLD polymers of second generation was the most

promising, where the obtained pyrene removal was 14% or 172 ppm of pyrene were present in the sample at the end of the process. With the preparations based on polymers of third and fourth generation, the degradation rates were 2 and 10%, respectively.

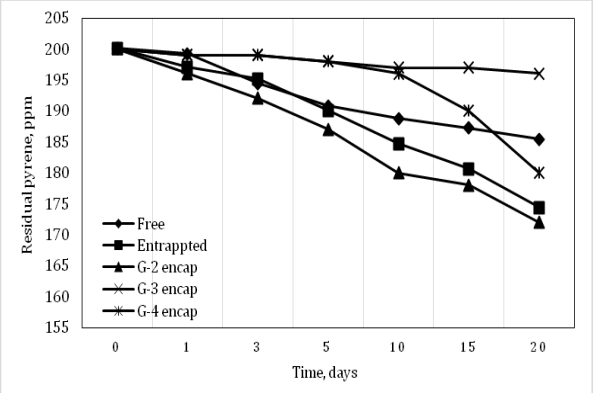

Fig. 2. Residual pyrene concentration

Compared to other studies, the obtained results initiate that with the implementation of some optimization of the process, higher degradation rates could be achieved. Laccase from *Lenzites betulinus* was immobilized for pyrene and benzo(a)pyrene degradation where over 80% and 40% degradation was achieved, respectively. The enzyme had a high degradation capacity which could be intensified by variation of the process parameters [18]. Deng *et al.* [19] applied immobilization of laccase on ferromagnetic nanoparticles for degradation of anthracene (3-ring PAH) and benzo(a)pyrene – a 5-ring PAH. The effects of time, pH, temperature, initial concentration of PAH and absence or presence of mediators were investigated. It was proven that the degradation efficiency could be significantly improved with the introduction of mediators into the reaction mixture. The immobilized preparation itself demonstrated a degradation rate of 39% for benzo(a)pyrene and in the presence of HBT, the degradation rate increased to 99.1%. In another study, laccase from *T. versicolor* was employed in the oxidation of 14 PAHs. The introduction of mediators in the degradation system led to a significant increase in the oxidation of pyrene to 48% whereas the degradation rate without a mediator was only 6% [20].

CONCLUSION

In the present study, the ability of free and immobilized laccase for the degradation of pyrene in aqueous medium was evaluated. The spectral data demonstrated changes in the absorbance values with time in the UV range, caused by structural changes in the pyrene molecule. For the first time, amphiphilic polymers were used for degradation of pyrene. The results indicated a potential for the development of a eco-friendly detoxifying technology. The application of DLD-based polymers for the degradation of water-insoluble xenobiotics in aqueous medium is a prospective field for new experiments. Further experiments are needed for the optimization of the degradation process regarding pH, temperature, duration of the process, presence of mediators, illumination, etc.

Acknowledgement: *This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students", approved by DCM # 577 / 17.08.2018". This study would not be possible without the generous contribution of Prof. Ivan Gitsov from the Department of Chemistry at SUNY College of Environmental Science & Forestry, Syracuse, NY, USA.*

REFERENCES

1. S. Mishra, Z. Lin, S. Pang, W. Zhang, P. Bhatt, S. Chen, *Front. Bioeng. Biotechnol.,* **9**, 632059 (2021).
2. V. K. Mishra, G. Singh, R. Shukla, *Clim. Change Agri. Ecosys*., **2**, 133 (2019).
3. L. B. Bertotto, T. R. Catron, T. Tal, *Neuro. Toxicol.,* **76**, 235 (2020).
4. N. Agrawal, P. Verma, S. K. Shahi, *Bioresour. Bioprocess.,* **5**, 11 (2018).
5. J. Merz, M. Dietz, Y. Vonhausen, F. Wöber, A. Friedrich, D. Sieh, I. Krummanacher, H. Braunschweig, M. Moos, M. Holzapfel, C. Lambert, T. Marder, *Chem. Eur. J.*, **26**, 438 (2020).
6. A. Saraswathy, R. Hallberg, *FEMS Microbiol. Lett*., **210**, 227 (2002).
7. M. Brazkova, G. Angelova, A. Krastanov, *Proc. Of the Fourth Intl. Conf. On Adv. Appl. Sci. Environ. Tech. – ASET* (2016).
8. A. Krastanov, R. Koleva, Z. Aleksieva, I. Stoilova, *Biotechnol. Biotechnol. Equip*., **27**, 4263 (2013).
9. H. Yemendzhiev, Z. Aleksieva, A. Krastanov, *Biotechnol. Biotechnol. Equip*., **23**, 1337 (2009).
10. I. Stoilova, G. Dimitrova, G. Angelova, A. Krastanov*, Bulg. J. Agric. Sci*., **23**, 988 (2017).
11. M. Fernandez, M. Sanroman, D. Moldes, *Biotechnol. Adv*., **31**, 1808 (2013).
12. M. Bradford, *Anal. Biochem.,* **72,** 248 (1976).
13. D. Daasi, S. Rodrigez-Couto, M. Nasri, T. Mechichi, *Int. Biodeterior. Biodegrad.,* **90**, 71 (2014).
14. I. Gitzov, K. L. Wooley, J. M. J. Fréchet, *Angew. Chem., Int. Ed. Engl*., **31,** 1200 (1992).
15. Z. You, H. Xu, K. J. Shah, J. Tao, D. Cai, *Chem. Eng. Trans*., **81**, 19 (2020).
16. J. Low, N. Abdullah, S. Vikineswary, *Res. J. Environ. Sci.,* **3**, 357 (2009).
17. M. Brazkova, M. Gerginova, I. Gitsov, A. Krastanov, in: Microbes in the Spotlight: Recent

Progress in the Understanding of Beneficial and Harmful Microorganisms, Mendez-Vilas (ed.), Brown Walker Press, 2016, p. 45.
18. X. Wang, S. Sun, Z. Ni, Z. Li, J. Bao, *J. Serb. Chem. Soc.,* **83**, 549 (2018).
19. J. Deng, H. Wang, H. Zhan, C. Wu, Y. Huang, B. Yang, A. Mosa, W. Ling, *Chemosphere*, **301**, 134753 (2022).
20. A. Majcherczyk, C. Johannes, A. Huttermann, *Enzyme Microb. Technol.,* **22**, 335 (1998).