Removal of Fluorene by native and modified by linear-dendritic copolymers Laccase from *Trametes versicolor*

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*Laccases are a part of the group of multicooper-containing oxidases, which in the last decades are object of intensive study due to their broad substrate specificity. Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic compounds which are abundant in the environment. Fluorene is a PAH, which is very toxic to aquatic organisms, causing long-term adverse effects in the aquatic environment. In the present study laccase production of *Trametes versicolor* was significantly enhanced by 2-methoxyphenol as inducing compound in the cultural media. Removal of fluorene was performed with crude laccase after the process of induction. The removal efficiency at the end of the process was 0.37 at 25°C and 0.98 for the process at 37°C. Laccase was isolated, purified and modified by using linear-dendritic-linear block copolymers. Spectral analysis show significantly more effective process of oxidation of fluorene by modified in comparison with the unmodified one.*

Keywords—Laccase, fluorene, oxidation, *Trametes versicolor*

I. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic compounds that are widely spread in the environment. The main sources of PAHs are the incomplete combustion of organic matter, internal combustion engines and different industrial processes [1]. PAHs can contain two or more fused benzene rings. In this regard they can be divided in two groups: PAHs with low molecular weight and PAHs with high molecular weight. The compounds from the first group have in their molecule up to three fused rings, and PAHs from the second group have four or more aromatic rings in their structure. PAHs are often found in groups in the environment. They are persistent, which makes them a concern for the environmental pollution as well as for animal and human health. Fluorene (fig 1) is formed of three fused aromatic rings. It forms white crystals with specific odor, similar to the one of naphthalene.

Fig.1 Structure of fluorene

Fig.2 Structure of dendritic-linear-dendritic copolymer

Fluorene is used as a chemical intermediate in many processes and in the formation of polyradicals for resins. Like most PAHs fluorine is used for dyes and plastic manufacturing and for the production of pesticides.

During the last decade laccases (p-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) are object of intensive research regarding to their capability to oxidize persistent environmental pollutants. Laccase production is defined mainly of the cultivation conditions and from the cultural media composition. *Trametes versicolor* produces its lignin degrading complex during the secondary metabolism and it is usually initiated by increased nitrogen concentration in the media or limited carbon source. Normally laccase is produced in small amounts from the white-rot fungi but the synthesis could be enhanced by the addition of inducers [2, 3]. Those inducers are mainly phenolic and aromatic or lignin related compounds. The addition of such substances leads to increasing the fields of application of *Trametes versicolor*'s laccase. Those enzymes have broad substrate specificity but their oxidation potential limits with only water soluble substrates. Linear-dendritic block copolymers have the ability to selectively adhere to various surfaces and they are have the quality to bind substantial amount of hydrophobic substrates in aqueous medium [4, 5]. The enzymatic nanoreactors used in the present study were formed by noncovalent envelopment of a glycoprotein by amphiphilic dendritic-linear-dendritic copolymers (fig.2).

The aim of the present work is to study the efficiency of the fluorene removal by crude laccase from *Trametes versicolor*, induced by 2-methoxyphenol in comparison with modified by linear-dendritic copolymers enzyme.

II. Materials and Methods

A. Fungal strain

The fungal strain *Trametes versicolor* 1 was provided by the Department of Biotechnology, University of Food Technologies – Plovdiv, Bulgaria. The strains is maintained on 1.5 % Czapek-Dox Agar and stored at 4°C.

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B. *Cultivation conditions. Enzyme assay.*

Czapek-Dox liquid media, containing sucrose – 30g/L; yeast extract – 5g/L; NaNO₃ – 2g/L; K₂HPO₄ – 1g/L; MgSO₄ – 0.5g/L; KCl – 0.5g/L and FeSO₄ – 0.01g/L was used for cultivation of *Trametes versicolor* 1. The pH prior sterilization was adjusted to 6.5 using 2% sulfuric acid.

Different concentrations in the range of 0-20 mM of 2-methoxyphenol were used in order of enhancing the biosynthesis of enzymes. The inoculation was made with 5% spores suspension of 7 days old culture from *Trametes versicolor* 1. The fermentation process was performed for 7 days at 28°C on a rotary shaker at 220 rpm.

After the end of the cultivation process the dry weight of the biomass, the protein concentration, the activity of the manganese-dependent peroxidase and laccase were determined. The dry weight was determined using RADWAG MA 50/1.R moisture analyzer where the biomass was dried at 105°C until constant weight.

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. The change of absorption values at 530 nm was monitored at 37°C for 5 minutes. One unit of enzyme activity corresponds to 0.001 change in OD 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, according to the method of Warshii et al. [6]. 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. One unit of enzyme activity was defined as the enzyme quantity required to oxidize 1μmol substrate for 1 minute at the reaction conditions. The total protein concentration was monitored using the "Bradford" method [7].

C. *Removal of fluorene. Extraction. HPLC analysis.*

After the end of the fermentation the biomass was separated from the media by centrifugation at 6000 rpm for 5 minutes. The cultural broth was used as medium for removal of fluorene. In 10 mL cultural broth, fluorene with concentration of 200 mg/L was added in dry form. The process was maintained at two different temperatures - 25°C and 37°C with duration of 15 days. Concentration of residual fluorene and enzyme activities were analyzed at 1st, 2nd, 3rd, 5th, 10th, 12th and 15th day.

Fluorene was extracted from the cultural broth using toluene as extracting agent. After triplicate extraction the crude extracts of organic phase were dried by adding of Na₂SO₄. Then the samples were evaporated under vacuum, resuspended in acetonitrile 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 at least three times and the mean was calculated.

The removal of fluorene (R, %) and the removal efficiency (η) were calculated using the formulas below:

$$R = \left[\frac{(C_0 - C)}{C_0} \right] * 100, \%$$

$$\eta = 1 - \left(\frac{C}{C_0} \right)$$

C₀ – initial concentration of fluorene, mg/L

C – concentration of fluorene on the state, mg/L

D. *Isolation and purification of laccase*

After the end of the fermentation process the cultural broth, containing the enzyme was filtered using glass fiber filters, brand G-6, followed by ultrafiltration with was performed using Milipore Prep/Scale-TFF ultrafiltration unit and a 400 mL Amicon stirred pressure cell, equipped a polyethersulfonate ultrafiltration membrane with 30000 Da molecular cutoff. Size exclusion chromatography was the final step of the purification process, were Sephacryl 200-HR was used. The blue colored fractions were collected, combined and concentrated again for gain of relatively homogeneous enzyme.

E. *Preparation of Laccase complex with the block copolymers*

The dendritic-linear-dendritic copolymers [G-2] – PEG5k – [G-2], [G-3] – PEG11k – [G-3] and [G-4] – PEG11k – [G-4] were synthesized from reactive poly(benzyl ether) monodendrons of second, third and fourth generation by coupling with preformed poly(ethylene glycol)s of molecular weight of 5000 Da or 10800 Da [8]. They were generously provided by the Department of Chemistry of SUNY College of Environmental Science & Forestry, Syracuse, NY, USA.

The solution of the purified enzyme (1mL, 2mg/mL protein concentration) was combined with 0.007 g of dendritic-linear-dendritic copolymers in dry form. Deionized water was added to make a final volume of 10 mL after which the mixture was stirred on 3D rotary shaker at 4°C for 12 h follow by stirring at room temperature for 8 h.

Fluorene was added in 200 mg/L concentration in 10 mL of the mixture containing the modified in copolymers enzyme. Experimental tubes were then placed on rotary shaker at 37°C.

F. *Spectral analysis*

The spectral analysis were performed on SpectroStar Nano UV-vis spectrophotometer (BMG Labtech) at room temperature with a scan speed of 1200 nm/min over a range of 220 – 950 nm, with 2 nm resolution. A COSTAR 96-well UV plate was used and 200 μl of the reaction mixture were taken for scanning.

III. Results and discussion

A. Induction of enzyme synthesis

The inducing substance, 2-methoxyphenol, was used in increasing concentration from 1 mM to 20 mM. The dry weight of the biomass was determined after cultivation at each concentration. The results obtained showed that there was significant decrease in biomass concentration where the 2-methoxyphenol concentration was over 4 mM (fig.3). Nevertheless there is no correlative connection between the biomass and the laccase gain. The analysis of the laccase activity after each induction process showed that 6 mM concentration of the inducing compound is optimal for producing enzyme with maximum activity (fig.4). The activity of MnP is also optimal at 6mM concentration of 2-methoxyphenol. The enzyme is stable at lower concentrations of the substance (fig.5), but concentrations over 8mM lead to sudden decrease in the activity of the MnP, as well as in the laccase activity. Compared with the results of previous research, [2], 2-methoxyphenol is the best inducer of the enzymes synthesis. The increase of the laccase activity was nearly 10 folds from 668 U/mL in the cultural broth without inducing component to 6468 U/mL in the cultural broth containing 6mM 2-methoxyphenol. The activity of MnP was increased with 32.4% from 52.5 U/mL to 69.5 U/mL.

Fig.3 Effect of 2-methoxyphenol on biomass gain

Fig.4 Effect of 2-methoxyphenol on laccase activity

Fig.5 Effect on 2-methoxyphenol on MnP activity

Therefore further in the experiments 6mM 2-methoxyphenol was used as inducer.

B. Fluorene removal

Trametes versicolor has the ability to degrade persistent environmental pollutants using its ligninolytic enzymatic system. Laccase is known to be able to oxidize various phenolic compounds, sometimes with the help of mediators [9]. The medium in which the experiment took place contained crude laccase and MnP (cultural broth, centrifuged at 6000 rpm for separation of the biomass), and fluorene, added in dry form, in concentration 200 mg/L. Fig. 6 shows the removal of fluorene (R) during the experimental procedure at 25°C and 37°C. On the first day of the experiment at 25°C, a residual fluorene concentration of 145 mg/L was detected. The concentration of residual fluorene in the samples placed at 37°C was 125 mg/L.

Until the 15th day there was no significant change in the residual fluorene detected at 25°C. The highest removal rate was on the last day and it equals to 37% or 75 mg/L fluorene removed. The data from the process carried out at 37°C was impressive. After 24h at the reaction conditions 75mg/L were removed and the oxidation continued to the 15th day where the removal rate was 98%. The removal efficiency at the end of the process was 0.37 for the process at 25°C and 0.98 for the process at 37°C. In the control samples fluorene concentration remain constant during the whole process which gave us the clue that the decrease in the concentration of fluorene is only due to the oxidative activity of the enzymes.

Fig.6 Fluorene removal by crude laccase and MnP, and laccase activity at different temperatures during the experiment

Laccase activity was also monitored during the process. The enzyme was active until the end of the process but lost 50% of its initial activity by the 5th day at 37°C and by the 10th day at 25°C. Despite the decrease of the laccase activity, the fluorene removal continues. This is due to the fact that the oxidation processes that take place in fluorene removal have chain-like characteristics. In this process the oxidized molecules continue to oxidase more fluorene molecules leading to increasing of the removal efficiency. The high removal rate is also a result of the lignin-degrading enzymatic complex activity, combined with the optimal temperature for the laccase action. The removal process took place in aquatic media. To our knowledge there is no research based on the fluorene removal with the usage of crude enzyme solution from *Trametes versicolor*. Akdogan *et al.* [10] used mediums containing different saline concentrations and *Trametes versicolor* strain for fluorene removal. The highest biodegradation rate was 54% and it was detected when an initial concentration of 5 mg/L fluorene was used. In the present study 98% removal of fluorene, with initial concentration of 200 mg/L, from the reaction media was achieved for the period of 10 to 15 days. This successful results is due most likely to the combination of the lignin-degrading complex (laccase, MnP) activity and the optimal temperature.

C. Monitoring of fluorene transformation by modified laccase

The goal of this study is to illustrate the outlined approach with the enzyme laccase and amphiphilic block copolymers containing linear poly(ethylene glycol), PEG and dendritic poly(benzyl ether)s. To our knowledge, the use of laccase-normal micelle complexes for the oxidation of fluorene in aqueous medium has not been reported. In this study we explore the catalytic activity of laccase from *Trametes versicolor* 1 embedded into normal micelles formed by a dendritic-linear-dendritic copolymers (fig.2). The experiment regarding laccase modification were performed with single laccase solution and the results observed could be due solely to the influence of the reaction conditions. The reaction mixture is formed by adding the components in specific order as follows: (1) enzyme in buffer solution, (2) copolymer in dry form and (3) fluorene in dry form. If different order of addition is applied the adhesion of the copolymers to the enzyme will be prevented and this will result in forming either empty copolymer micelles or substrate loaded ones.

An initial quantity of 200 mg/L of fluorene was introduced into the samples. Spectral analysis took place at every hour during the first five hours, and every 24 hours after that until the 48th hour of the experiment. The biotransformation of fluorene is followed by UV-Vis spectroscopy (fig.7). Parallel measurements without the linear-dendritic copolymer are also made under identical reaction conditions. It is seen that the spectra changes in the different reaction mixtures over time. The spectroscopic analyses reveal that the mixtures containing laccase modified with [G2]-PEG5k-[G2] show a broad absorbance in the region 220 – 420 nm increasing with time (fig.7b). It is clear that the most significant spectral changes occur in the mixture, containing [G-2] – PEG5k – [G-2] polymer.

Those changes are almost instantly starting with the mixing of the components. In comparison with the other graphs here dynamic and rapid changes in the spectra were observed for the first 5 hours of the experiment, but the biggest difference between the spectral curves was observed between the 5th and 24th hour. At fig. 5 c) where [G-3] – PEG11k – [G-3] polymer was used spectral changes also occur, but in not so dynamic way as in the previous graph. The changes during the first five hours were not so dynamic, but are clearly visible. Again the biggest change in the spectral curves was observed at the 24th and the 48th h. When the [G-4] – PEG11k – [G-4] polymer was used the only significant change appears at the spectral curve of the 48th h. Both figures c) and d) are similar to a) where the enzyme solution wasn't modified with polymer. This means that the modification with [G-2] – PEG5k – [G-2] is the most successful one.

Fig.7 UV-Vis spectra of aqueous reaction mixture containing fluorene and: a) native enzyme solution; b) enzyme encapsulated with [G-2] – PEG5k – [G-2] polymer; c) enzyme encapsulated with [G-3] – PEG11k – [G-3] polymer and d) enzyme encapsulated with [G-4] – PEG11k – [G-4] polymer

The observed changes in the spectral curves are result of oxidation processes that took place in the reaction mixture. During the oxidation of fluorene probably new compounds were formed. The control samples contained native enzyme, modified enzyme or fluorene with polymer and showed no changes in the spectral curves during the experiment. The most significant spectral changes were observed at 400 nm. Spectral data showed (fig.8) there is most likely no difference in the structure of the compounds, formed due to the oxidation processes when native enzyme solution and [G-4] – PEG11k – [G-4] polymers were used. When [G-3] – PEG11k – [G-3] and [G-2] – PEG5k – [G-2] polymers were tested there is significant change in the absorbance values. Those differences are probably due to the intense oxidation processes leading to formation of different intermediate products with different molar attenuation coefficient.

Fig 8. Changes in the absorbance at 400 nm of different enzyme preparations

The process of envelopment of the laccase with linear-dendritic copolymers is a way for the enzyme to be involved of oxidation processes of insoluble persistent phenolic and aromatic environmental pollutants in aquatic media. With the modification of the crude laccase solution the time needed for fluorene removal/biotransformation was reduced from 15 days to 48 h. This approach could be successfully used for the needs of green chemistry and for the removal of other PAHs and insoluble hazardous pollutants.

Further analysis are necessary for determination the exact pathway of fluorene oxidation and also determination the removal rate of the compound when modified enzymes are used.

iv. Conclusions

In this study a successful method for oxidation of fluorene using cultural broth with induced laccase activity was presented. To our knowledge this is the first research regarding fluorene removal/biotransformation in aquatic media without the presence of mediators. The removal rate of fluorene from the reaction mixture after 15 days at 37°C was 98%. The data of the current investigation show that laccase surface-modified with the amphiphilic copolymer [G2]-PEG5k-[G2] is capable of oxidative transformation of highly hydrophobic PAHs (fluorene) without the presence of mediators. When the [G-2] – PEG5k – [G-2] linear-dendritic linear copolymer was used for modification of the purified enzyme, the changes in the spectral curves were most significant. Those changes most probably are due to oxidation processes in the mixture containing fluorene and

modified enzyme. The time needed for fluorene removal/biotransformation in this case was reduced to 48 h.

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