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Azonaphthalene dyes decolorization and detoxification by laccase from *Trametes versicolor*

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ABTS, 2,2´-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
HBT, 1-hydroxybenzotriazole
MW, molecular weight

Abstract

The aim of the present study was to investigate the dye decolorization ability of laccase from *Trametes versicolor*. Five azonaphthalene dyes (Acid Violet 7, Acid Red 1, Allura Red AC, Orange G and Sunset Yellow FCF) were used to evaluate dye decolorization. Laccase from *T. versicolor* is capable of decolorizing dyes, namely Acid Violet 7 (53.7±2.3 %) and Orange G (46.0±2.2 %). The less effective ability of laccase was observed at the decolorization of other selected dyes (6.9 – 18.6 %). The presence of redox mediator (1-hydroxybenzotriazole) increased decolorization percentage for all tested dyes (≥ 90.5 %). Toxic effect of azo dyes and their degradation products after laccase treatment was observed on the growth of selected bacteria (*Micrococcus luteus*, *Bacillus subtilis*, *Pseudomonas syringae* and *Escherichia coli*), yeasts (*Candida parapsilosis* and *Saccharomyces cerevisiae*) and algae (*Chlorella vulgaris* and *Microcystis aeruginosa*). It was confirmed that degradation products showed lower inhibition effect compared to initial dyes. These findings suggest that laccase from *T. versicolor* are able to decolorize and detoxify selected azonaphthalene dyes.

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Introduction

Azo dyes represent a group of synthetic dyes used in industrial processes for their stability and colour intensity (Gomez *et al.* 2013; Rovina *et al.* 2016). This group belongs to a large class of synthetic dyes containing one or more azo bonds (-N=N-). These bonds link various aromatic ring structures (benzene, naphthalene).
Azonaphthalene dyes have naphthalene ring with delocalized conjugated bonds of carbon atoms stabilise total structure of azo dye (Zhu *et al.* 2012). Moreover, functional groups present in their structure are responsible for various physical properties such as solubility, lipophilicity or absorption (Legerská *et al.* 2016; Da Costa *et al.* 2017). Approximately 2 – 50 % of synthetic dyes used in industrial dyeing operations have been discharged into wastewater (Sarkar *et al.* 2017).
Moreover, many works confirmed that azo dyes are toxic or carcinogenic for various organisms including human (Yang *et al.* 2009; Gholami-Borujeni *et al.* 2011; Axon *et al.* 2012; Nath *et al.* 2016). Therefore, the elimination azo dyes from water systems by appropriate methods is essential for prevention of the environment contamination. For azo dye removal, some chemical and physico-chemical methods have been traditionally used. Their disadvantages include sludge formation (Zhu *et al.* 2012; Lau *et al.* 2014; Youssef *et al.* 2016), high operating costs and toxic degradation products (Liakou *et al.* 1997; Lopez *et al.* 2004; Linley *et al.* 2012). Biological elimination of synthetic azo dyes is environmentally friendly and relatively

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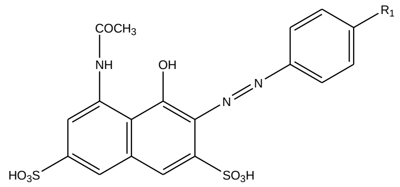
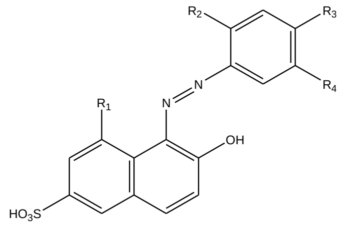
Table 1. Characteristics of azonaphthalene dyes used in this study.

***α*-naphthol dyes**



	CAS number	R_1	λ_{max} [nm]	MW [g/mol]
Acid Violet 7	4321-69-1	–NH–$COCH_3$	519	566.8
Acid Red 1	25956-17-6	–	531	537.8

***β*-naphthol dyes**



	CAS number	R_1	R_2	R_3	R_4	λ_{max} [nm]	MW [g/mol]
Allura Red AC	25956-17-6	–	–OCH_3	–SO_3H	–CH_3	493	496.4
Orange G	1936-15-8	–SO_3H	–	–	–	479	452.8
Sunset Yellow FCF	2783-94-0	–	–	–SO_3H	–	480	452.4

inexpensive. Moreover, the biological methods do not form any toxic degradation products (Sathe *et al.* 2015; Legerská *et al.* 2016, Chmelová and Ondrejovič 2016). Useful enzymes for this purpose seem to be azoreductases (EC 1.7.1.6), laccases (EC 1.10.3.2) and peroxidases (1.11.1.x) (Viswanath *et al.* 2014; Singh *et al.* 2015). In contrast to azoreductases and peroxidases, laccases catalyse dye decolorization in the presence of oxygen. Consequently, laccase dye decolorization offers more efficient degradation, low cost of enzymatic process and the production of non-toxic compounds comparing to other above-mentioned enzymes (Casas *et al.* 2007; Chhabra *et al.* 2015).

While different organisms produce laccases including bacteria, fungi, plants and insect, the most perspective producers are white-rot fungi (Viswanath *et al.* 2014; Singh *et al.* 2015, Hazuchová *et al.* 2017) producing laccases with high redox potential (Baiocco *et al.* 2003; Pang *et al.* 2015). For example, laccases from *Trametes versicolor* have the redox potential approximately 785 mV, which allows oxidation of hardly-degradable organic compounds including various groups of dyes (Kurniawati and Nicell 2008; Legerská *et al.* 2018). Moreover, laccases produce non-toxic degradation products, which saprophytes can use as carbon source (Gavril and Hodson 2007; Selvam *et al.* 2012).

Therefore, the aim of this study was to evaluate potential of laccase from *T. versicolor* for decolorization and detoxification of synthetic dyes from the group of *α*-naphthol (Acid Violet 7, Acid Red 1) and *β*-naphthol (Allura Red AC, Orange G, Sunset Yellow FCF) azo dyes.

Experimental

Microorganisms

Bacterial species (*Micrococcus luteus* CCM 1569, *Bacillus subtilis* CCM 2218, *Pseudomonas syringae* CCM 2114 and *Escherichia coli* CCM 7929) and yeast species (*Candida parapsilosis* CCM 8186, *Saccharomyces cerevisiae* CCM 8191) were purchased from Czech Collection of Microorganisms (Brno, Czech Republic).

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The algae *Chlorella vulgaris* H 1993 was purchased from Culture Collection of Algae (Prague, Czech Republic) and *Microcystis aeruginosa* PCC 7806 was obtained from Culture Collection of Cyanobacteria and Algae (Brno, Czech Republic).

Dyes and chemicals

Laccase from *Trametes versicolor* and selected naphthalene azo dyes (Table 1) were obtained from Sigma-Aldrich (Germany). Culture media were supplied by Biolife (Italy). All other chemicals were purchased from Mikrochem (Slovak Republic).

Decolorization of azonaphthalene dyes by laccase

Dye decolorization by laccase from *T. versicolor* was spectrophotometrically determined in the range of 300 – 700 nm (Spectrophotometer V-1600PC, VWR, Germany) during 5 days with or without the redox mediator 1-hydroxybenzotriazole (HBT). The reaction mixtures contained 50 mg/L of azonaphthalene dye in 0.1 mol/L phosphate buffer (pH 3.0), and laccase from *T. versicolor* with enzyme activity of 1.0 U/mL mixed in ratio 3 : 1 (*v*/*v*). The effect of 1.0 mmol/L HBT was also tested. Control samples were run without the addition of laccase. The decolorization percentage was determined as follows:

$$decolorization\ [\%] = [(A_0 - A_t)/A_0]*100,$$

where A_0 is the initial absorbance and A_t is the absorbance of enzymatic reaction at a certain time of laccase treatment.

Toxicity test

The effect of laccase treatment on toxicity of selected azonaphthalene dyes, namely Acid Violet 7 and Orange G, were evaluated. The applied dye concentration range was 0.04 – 5.0 g/L. The growth inhibitions of selected microorganisms (bacteria, yeasts and algae) were assayed in the microtiter plates by dilution method during 48 and 72 h at 30 °C spectrophotometrically at 690 nm. The results were expressed as minimum inhibitory concentration (MIC), which completely inhibits the growth of microorganism.

Production of chlorophylls and biomass was monitored for algae (*C. vulgaris*, *M. aeruginosa*) in media with selected azo dyes or their degradation products after laccase treatment. The cultivation was performed at laboratory temperature with 14 : 10 h light and dark photoperiod. After 30 days of cultivation, the concentration of chlorophylls *a* and *b* in algal biomass were expressed according Sumanta *et al.* (2014) as follows:

$$chlorophyll\ a = 16.72A_{665nm} - 9.16A_{652nm}$$

$$chlorophyll\ b = 34.09A_{654nm} - 15.28A_{665nm}$$

The results were expressed as the inhibition percentage toward the control without synthetic dye or degradation products of dye after laccase treatment. The inhibition of biomass formation was calculated as follows:

$$biomass\ inhibition\ [\%] = [(B_c - B_d)/B_c]*100,$$

where B_c is biomass weight of control sample without azo dye and B_d is biomass weight of sample with selected azo dye. The inhibition of chlorophyll production was calculated as follows:

$$chlorophyll\ inhibition\ [\%] = [(C_c - C_d)/C_c]*100,$$

C_c is the concentration of selected chlorophyll *a* or *b* in control sample and C_d is the concentration of selected chlorophyll *a* or *b* in sample with azo dye or degradation products after laccase treatment.

HPLC analysis

The degradation mixtures of selected azonaphthalene dyes (Acid Violet 7 and Orange G) were analysed with high performance liquid chromatography HPLC (Agilent Technologies 1200 Series, USA) on C18 column (3.5 µL, 3.0 mm x 100 mm) using mobile phase A (0.1 % (*v*/*v*) aqueous solution of formic acid) and B (0.1 % (*v*/*v*) methanolic solution of formic acid). The gradient program was set as follows: 0 – 2 min = 0 – 20.0 % B, 2 – 15 min = 20.0 – 95.0 % B, 15 – 20 min = 95.0 % B. The flow rate was 0.7 mL/min and the absorbance of eluted compounds was measured at 254 nm.

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Results and Discussion

Decolorization of selected azonaphthalene dyes

Laccases are often studied for their potential of dye decolorization. Five azonaphthalene dyes, Acid Violet 7, Acid Red 1, Allura Red AC, Orange G and Sunset Yellow FCF, were used to evaluate the dye decolorization efficiency of laccase from *T. versicolor* (Fig. 1). Laccases from *T. versicolor* were able to decolorize the *α*-naphtol dye Acid Violet 7 (53.7±2.3 %) (Fig. 1-A). The *α*-naphtol dye Acid Red 1 was decolorized by laccase less effectively (16.7±0.1 %) (Fig. 1-B). Dye degradation by laccase is influenced by chemical structure of dye itself, differences in electron

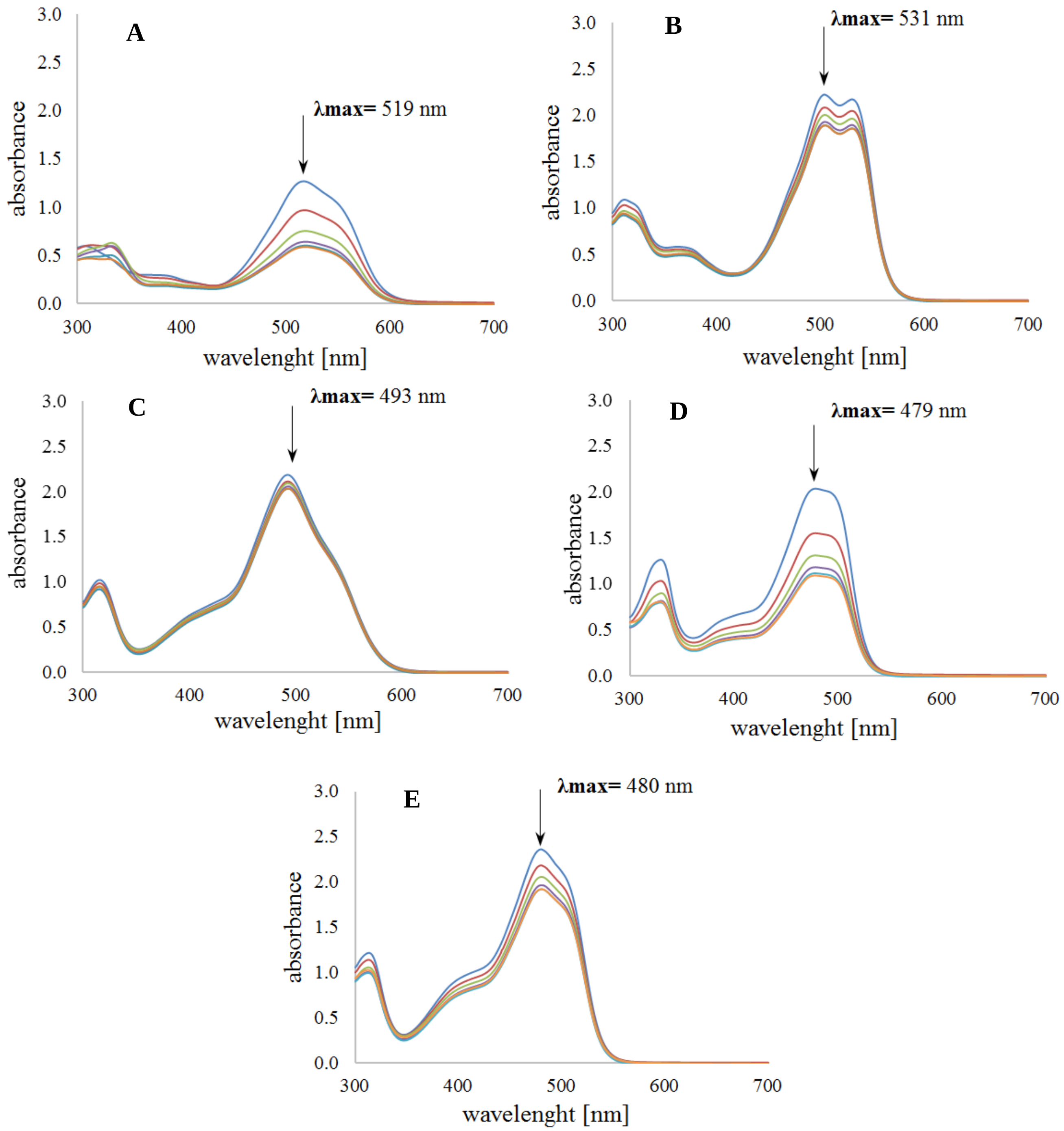

Fig. 1. UV-VIS spectrum of the reaction mixture of the naphthalene dye **A** – Acid Violet 7, **B** – Acid Red 1, **C** – Allura Red AC, **D** – Orange G and **E** – Sunset Yellow FCF decolorized with laccase from *Trametes versicolor* during 5 days at pH 3.0 and 22 °C (▬ 0. day, ▬ 1. day, ▬ 2. day, ▬ 3. day, ▬ 4. day, ▬ 5. day).

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Table 2. The effect of 1-hydroxybenzotriazole (HBT) on laccase catalysed decolorization of selected azo dyes.

Azo Dyes	**Decolorization [%]**	
	Laccase without HBT	**Laccase-HBT mediated System**
***α*-naphtol dyes**		
Acid Violet 7	53.7±2.3	97.1±0.3
Acid Red 1	16.7±0.1	95.0±0.5
***β*-naphtol dyes**		
Allura Red AC	6.9±0.2	95.8±0.1
Orange G	46.0±2.2	96.8±0.7
Sunset Yellow FCF	18.6±1.2	95.0±1.5

distribution, number of dissociating groups and steric barriers (Hsueh *et al.* 2009). The poorest was decolorization of Acid Red 1 probably because of the presence of -$COCH_3$ group bonded to the naphtol ring of Acid Red 1. Similarly, other authors described partial decolorization of these dyes. Zhang *et al.* (2006) tested the degradation ability of laccase from *Panus rudis* for Acid Violet 7 and reported decolorization of 26.0 %. This substituent belongs to electron-withdrawing group making a ring less susceptible to enzymatic oxidation (Suzuki *et al.* 2001). In contrast to our results, Zhou *et al.* (2017) observed higher decolorization of Acid Red 1 (69.7 %) after enzymatic reaction with laccase from *Bacillus pumilus* W3. The efficiency of the decolorization process, therefore, is obviously influenced by the choice of producer. Different producers produce laccase with various redox potential resulting in variable decolorization efficiency (Baiocco *et al.* 2003; Pang *et al.* 2015).

Of the *β*-naphtol dyes, the highest decolorization by *T. versicolor* laccases was showed for Orange G (46.0±2.2 %) (Fig. 1-D). The other *β*-naphtol dyes, Sunset Yellow FCF and Allura Red AC, were decolorized less effectively (18.6±1.2 % and 6.9±0.2 %, respectively) (Fig. 1-C,E). The efficient decolorization of Orange G was probably caused by the presence of two sulfo groups linked to the naphtol ring (Table 1), which allows simple degradation of dye chromophore. Sunset Yellow FCF has the similar structure as Orange G, however, the presence of single –SO_3H group on the benzene ring significantly reduces structure stabilization. The lowest decolorization was observed in the mixture of Allura Red AC and laccase from *T. versicolor* (6.9±0.2 %). The presence of methyl- and methoxy groups on the benzene ring restricted decolorization. These groups stabilize the dyes and prevent their effective oxidation by laccase (Chivukula and Renganathan 1995; Hsueh *et al.* 2009).

The dye decolorization by laccases can be enhanced using low molecular compounds, also called redox mediators. In our work, we tested the effect of 1-hydroxybenzotriazole (HBT) as synthetic redox mediator on laccase catalysed decolorization of selected azo dyes. The results are shown in Table 2.

The presence of HBT increased the degree of decolorization of all tested dyes (Table 2), while the decolorization percentages varied in the range of 95.0 – 97.1 %. Forootanfar *et al.* (2016)

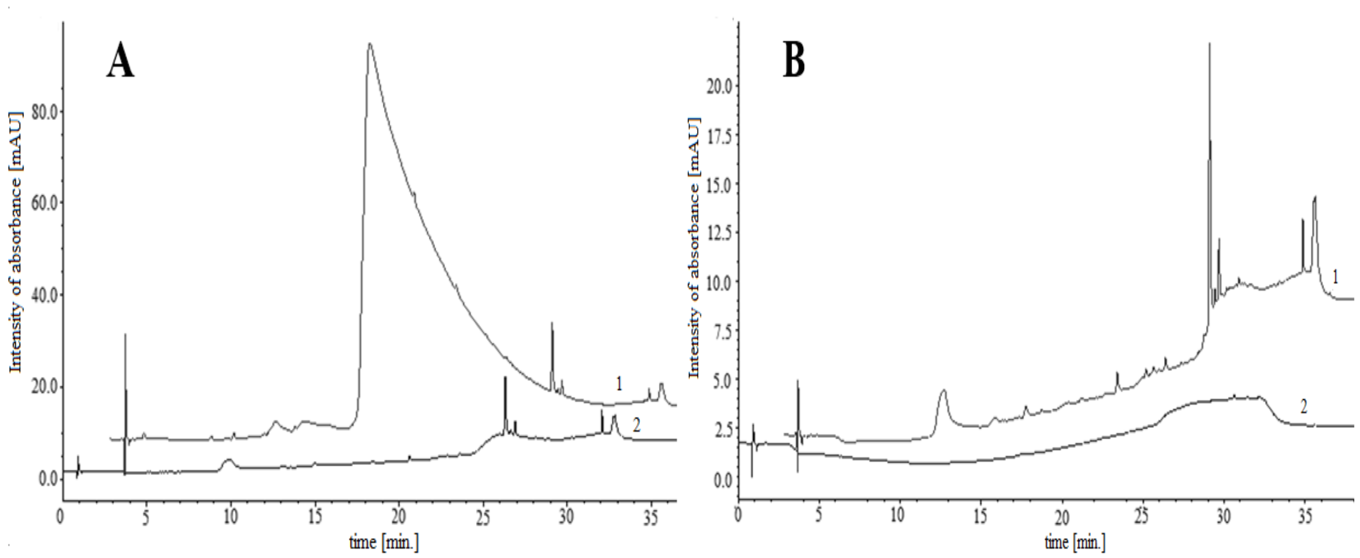

Fig. 2. HPLC elution profile of azonaphthalene dyes (1) (**A** – Acid Violet 7, **B** – Orange G) and their degradation products (2) obtained after treatment with laccase from *T. versicolor*.

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found that the presence of redox mediator (HBT) with laccase from *Paraconiothyrium variabile* increased the decolorization of azo dye and decreased the needed time for degradation. Wong and Yu (1999) reported that laccase from *T. versicolor* decolorized Acid Violet 7 more effectively in the presence of redox mediator (ABTS) in the reaction mixture. Furthermore, Zeng *et al.* (2011) reported decolorization of Acid Red 1 only in the laccase-HBT mediated system. It seems that the selection of appropriate redox mediator can increase the efficiency of decolorization process catalysed by laccases.

Degradation products analysis

Several authors (Yang *et al.* 2009; Gholami-Borujeni *et al.* 2011; Axon *et al.* 2012; Nath *et al.* 2016) describe the potential toxic effect of dye degradation products. Therefore, two best decolorized dyes were assessed for the effect of laccase treatment. Degradation products of synthetic dyes after enzymatic degradation can be mainly analysed by HPLC or GC-MS (Franciscon *et al.* 2012; Christiane *et al.* 2013; Yuan *et al.* 2016). In our work, HPLC was used as a tool for analysis of dye decolorization and detection of emerging products. The results are shown in Fig. 2.

HPLC analysis of Acid Violet 7 (Fig. 2-A) displayed a single peak at retention time 15.457 min. After laccase treatment, the loss of the main peak indicates decolorization of initial dye. Similarly, HPLC analysis of Orange G (Fig. 2-B) displayed peaks at 25.310 min, 26.888 min, 32.070 min and 32.839 min. In reaction mixture with degradation products after laccase catalysed reaction, non-detectable peaks were observed. Although, new peaks were not detected, disappearance of the main peaks in Acid Violet 7 (15.457 min) and Orange G (25.310 min) profiles point to the effectivity of laccase from *T. versicolor* to decolorization azonaphthalene dyes without production of other detectable compounds.

Toxicity tests

Azo dyes belong to toxic compounds with potential toxic effect on prokaryotic and/or eukaryotic organisms (Przystas *et al.* 2012; Lade *et al.* 2015). The loss of dye colour after laccase treatment indicates the breakdown of parent compound. However, degradation products can also be potentially toxic. Therefore, toxicity tests of selected azo dyes, namely Acid Violet 7 and Orange G, with the highest decolorization after laccase treatment were performed (Table 3).

The toxicity of Acid Violet 7 on the growth of all bacteria (*M. luteus*, *B. subtilis*, *P. syringae*, *E. coli*) and the yeast *C. parapsilosis* was observed at dye concentration of 5.0 g/L, except of *S. cerevisiae* (>5.0 g/L). Similarly, Mansour *et al.* (2010) recorded toxicity of Acid Violet 7; this azo Dye induced chromosomal aberrations, lipid

Table 3. The minimum inhibitory concentrations of azonaphthalene dyes to the growth of prokaryotic and eukaryotic organisms before and after laccase treatment.

	Minimum inhibitory concentration [g/L]			
	Acid Violet 7		Orange G	
	Synthetic Dye	Degradation Products	Synthetic Dye	Degradation Products
Bacteria				
M. luteus	5.0	-	5.0	-
B. subtilis	5.0	-	5.0	-
E. coli	5.0	-	-	-
P. syringae	5.0	-	-	-
Yeasts				
C. parapsilosis	5.0	-	-	-
S. cerevisiae	-	-	-	-

– without inhibitory effect on bacterial and yeast growth in concentration range of 0.04 – 5.0 g/L.

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Table 4. The inhibitory effects of Acid Violet 7 and Orange G and their degradation products after laccase treatment on the biomass and chlorophylls production of selected algae.

			Inhibitory rate [%]	
			C. vulgaris	*M. aeruginosa*
Acid Violet 7	Synthetic Dye	biomass	**37.8±3.2**	-
		chlorophyll *a*	14.5±2.8	**56.7±4.6**
		chlorophyll *b*	12.9±1.2	**56.1±3.7**
	Degradation Products	biomass	21.9±1.8	-
		chlorophyll *a*	6.9±0.4	-
		chlorophyll *b*	8.8±2.9	-
Orange G	Synthetic Dye	biomass	**30.3±4.9**	-
		chlorophyll *a*	14.8±2.1	**72.1±3.1**
		chlorophyll *b*	10.7±2.1	**71.2±4.7**
	Degradation Products	biomass	10.6±3.6	-
		chlorophyll *a*	-	-
		chlorophyll *b*	-	-

– without inhibitory effect to the growth of algae species.

peroxidation and cholinesterase inhibition in mouse bone marrow. On the other hand, in our study, Orange G inhibited only the growth of *M. luteus* and *B. subtilis* (Table 3), its growth inhibitory effect was not observed on other tested microorganisms. This lack of toxicity of Orange G is consistent with the results of Mariappan *et al.* (2003) for bacterial species *Pseudomonas sp*. SAC03, *Bacillus sp*. SAC01 and *Escherichia sp*. SAC01, and also Alcántara *et al.* (2017) for yeast species. Previous studies have indicated that the toxicity of azo dyes strongly depends on chemical structure, functional groups and the number of azo bonds (Costa *et al.* 2012; Legerská *et al*. 2016; Da Costa *et al*. 2017). This was also confirmed in this study showing the relatively higher toxicity of α-naphtol dye Acid Violet 7 comparing to β-naphtol dye Orange G. Even more importantly, laccase treatment resulted in degradation products with no toxicity on tested microorganisms (Table 3).

The presence of synthetic dyes in environment also affects the growth of photosensitive microorganisms. Therefore, we tested the impact of laccase-degraded dyes on growth of selected algae, namely *C. vulgaris* and *M. aeruginosa*. Contents of chlorophyll *a* and *b* were measured. The results are shown in Table 4.

The presence of Acid Violet 7 and Orange G in medium inhibited the growth of *C. vulgaris* as well as the production of chlorophyll *a* and *b* of both algae species (Table 4). In culture media with degradation products after laccase treatment, the reduction and even disappearance of toxicity effect were observed. Influence of azo dye on *C. vulgaris* growth was also studied in the work of Hernández-Zamora *et al.* (2014) who revealed significant decrease of biomass and chlorophyll production. El-Sheekh *et al.* (2017) confirmed the negative influence of azo dye Disperse Red BS on growth of *M. aeruginosa*.

Conclusions

The results from this study showed the ability of laccase from *Trametes versicolor* to decolorize synthetic dyes from model systems. As expected, decolorization was observed in all dye reaction mixture. Laccase from *T. versicolor* showed the highest decolorization with Acid Violet 7, the α-naphthol azo dye, and with Orange G, the β-naphthol azo dye while the addition of HBT increased the effectivity of dye degradation. HPLC analysis of decolorized solutions confirmed absence of distinguishable degradation products and complete loss of the main peak representing azo dye. The dye toxicity toward bacteria, yeasts and algae after laccase catalysed reaction was decreased. Our results show that laccases from the white rot fungus *T. versicolor* are suitable for decolorization and detoxification of azonaphthalene dyes, while generate environmentally friendly degradation products.

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