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Biodegradation of diclofenac with fungal strains

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Abstract: Diclofenac (2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid) is a non-steroidal anti-inflammatory drug. Due to excessive use of diclofenac, this drug has been detected in surface water, ground water and drinking water. In our study, four fungal strain *Trametes trogii*, *Aspergillus niger*, *Yarrowia lipolytica* and *Phanerochaete chrysosporium* were investigated in terms of diclofenac degradation potential. *Trametes trogii* was found to be the most efficient strain with 100% diclofenac degradation rate. Two hydroxylated diclofenac metabolites have been identified in culture medium. Crude laccase from *T. trogii* almost completely removed diclofenac with 97% removal in 48 h. We suggest that the degradation of diclofenac depends on the cytochrome P450 enzyme system and laccase activity. After 24 h incubation decrease in toxicity of diclofenac was confirmed by Microtox test.

Introduction

In recent years, the presence of micropollutants such as pharmaceuticals, industrial chemicals, personal care products and many other chemical compounds in the aquatic environment have become a significant problem worldwide (Luo et al. 2014). Due to excessive usage of pharmaceuticals in human and veterinary medicine, pharmaceuticals have been detected in surface water, ground water, and even in drinking water (Domaradzka et al. 2015).

Disposal of unused drugs, pharmaceutical production facilities, hospital wastes, human excretions and veterinary applications are the origin of the pharmaceutical pollution. If they are not eliminated properly in waste water treatment plants, pharmaceutical active compounds can enter aquatic systems (Kümmerer 2001, Heberer 2002, Fent et al. 2006).

Diclofenac (2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid) is a non-steroidal anti-inflammatory drug which is widely used for analgesic and antipyretic properties. The global usage of diclofenac is about 1000 ton per year (Zhang and Geissen 2010). According to Directive 2008/105/EC of European Parliament and of Council, diclofenac should be included in the first watch list (Official Journal of the European Union). Diclofenac has been detected in ground waters, surface waters, and even in drinking waters in up to µg L^{-1} (Zhang et al. 2008). Oaks et al. reported that diclofenac residues were responsible for death of vultures in Pakistan and India (Oaks et al. 2004). Also, diclofenac has been classified as dangerous for aquatic life; the effects of diclofenac have been more extensively studied in fish after prolonged exposure. Cytological alterations in liver, kidneys, and gills were observed in rainbow trout (Carlsson et al. 2006).

Various psychochemical methods such as advanced oxidation, photolysis, ozonation, ultrasonic irradiation, photo-fenton degradation etc. have been used to degrade diclofenac (Pérez-Estrada et al. 2005, Gromadzka and Nawrocki 2006, Trapido et al. 2012, Yu et al. 2013, Nie et al. 2014). Also, besides these methods, many researchers achieved degradation of diclofenac using biological methods (Hata et al. 2010, Zhang and Geissen 2010, Marco-Urrea et al. 2010b, Langenhoff et al. 2013). Biological methods are more robust and economically feasible than oxidation processes (Langenhoff et al. 2013).

Trametes trogii and Phanerochaete chrysosporium are white-rot fungi. White rot fungi secrete extracellular enzymes that are responsible for lignin degradation (Pointing 2001) Previous studies showed that white rot fungi *Trametes versicolor* can degrade various non-steroidal, anti-inflammatory drugs such as naproxen, ketoprofen and ibuprofen (Marco-Urrea et al. 2009, Marco-Urrea et al. 2010c, Marco-Urrea et al. 2010a).

Yarrowia lipolytica is a non-pathogenic yeast that is used for lipase, protease, RNAase, peach flavor and citric acid production (Destain et al. 1997, Schrader et al. 2004, Rymowicz et al. 2010). *Aspergillus niger* is an important filamentous fungus that is used in biotechnology for enzyme production (Schuster et al. 2002).

The main aim of this study is to determine diclofenac removal capabilities *of Phanerochaete chrysosporium*, *Trametes trogii*, *Aspergillus niger* and *Yarrowia lipolytica*.

Materials and Methods

Chemicals

Diclofenac sodium (CAS NO: 15307-79-6) was obtained from Sigma-Aldrich.

Fungus
In this study *Trametes trogii* (*Funalia trogii* ATCC 200800), *Aspergillus niger* NRRL 328, *Yarrowia lipolytica* NBRC 1658 and *Phanerochaete chrysosporium* ME 446 were used. Cultures of fungi were maintained by subculturing on potato dextrose agar at 30°C. Fungal cultures were kept at +4°C for up to 20 days.

Culture medium
Biodegradation experiments were performed in 250 ml Erlen--meyer flasks containing 100 ml culture medium. The culture medium was composed of (L^{-1}) 20 g glucose, 2 g NH_4NO_3, macro and micronutrients (Aktaş et al. 2001). All mediums pH were adjusted to 4.7 using 0,1 N HCL.

Degradation experiments
One milliliter of suspended mycelia of *Trametes trogii* and one milliliter of spore suspension of *Phanerochaete chrysosporium* (1 mL; 0.5 absorbance unit at 650 nm) were inoculated into 250-mL flasks. Cultures of these fungi were precultured for 7 days at 30°C (150 rpm). One milliliter of *Yarrowia lipolytica* cell suspension (1 mL: 1 absorbance unit at 650 nm) and one milliliter of *Aspergillus niger* spore suspension (1 mL; 1.2×10^6 spore) were inoculated into 250-mL Erlenmeyer flasks and then cultures were precultured for 3 days at 30°C (150 rpm). After preincubation periods, 1-mL stock solution of diclofenac in ethanol was added into flasks to give desired final concentration of pharmaceutical (50 mg L^{-1}). To avoid photo transformation reactions, all the experiments were carried out in the dark. Each experiment was prepared triplicate, control groups included abiotic and heat killed organisms.

LC/MS analysis
The LC/MS spectra were taken on a Waters Micromass ZQ connected with Waters Alliance HPLC, using ESI(+) method, with C-18 column. The HPLC of LC/MS was carried out on a column XTerra®MS C-18 (4.6 mm × 250 mm, 5 µm) with Water : MeOH : acetonitrile: % 0.1 HCOOH in acetonitrile (10:15:65:10) as mobile phase. The flow rate was 0.55 mL/min, the injection volume was 4 µL and the running time was 10 min. The eluate was monitored by a photo-diode array detector at 254 nm. The analytical condition of mass was as follows: capillary voltage: 3.71 kV, cone voltage: 29 V, source temperature: 100°C : desolvation temperature: 350°C.

Enzyme assay
Laccase activity was determined by measuring oxidation of ABTS (2,20-azinobis-(3-ethylbenzothiazo- line-6-sulphonic acid) at 420 nm in pH 4.5 100 mM acetate buffer (Bourbonnais and Paice 1990). Lignin peroxidase activity was assayed by oxidation of veratryl alcohol to veratrylaldehyde at 310 nm in pH 3 100 mM sodium tartrate buffer (Arora and Gill 2001). Manganese peroxidase activity was determined by formation of Mn^{+3} in pH 5 sodium tartrate buffer at 238 nm (Camarero et al. 1999). Tyrosinase activity was assayed using L-Dopa as a substrate. Dopachrome formation was monitored spectrophotometrically at 475 nm (Majidi and Aksöz 2013). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of product per minute.

Purified tyrosinase and laccase
Commercial tyrosinase (mushroom) and laccase (*Trametes versicolor*) were obtained from Sigma-Aldrich. Laccase mediated degradation studies were performed in 250 ml Erlenmeyer flasks at pH 4.5 (final activity: 2.4 U/ml). Tyrosinase mediated degradation studies were performed in 250 ml Erlenmeyer flasks at pH 7 (final activity: 2 U/ml).

Crude laccase
Crude laccase was prepared as follows. One milliliter of suspended mycelia of *Trametes trogii* was inoculated culture medium incubated for 5 days at 30°C, 150 rpm, pH 4.5. After incubation period *Trametes trogii* culture was separated from biomass via centrifuging. The obtained cell free culture supernatant was used as a crude laccase source.

Toxicity test
Microtox bioassay (MicroBioTests Inc.) was used to monitor changes in diclofenac toxicity. Microtox is an in vitro testing system which uses bioluminescent bacteria *Vibro fischerii* to assess toxicity. Toxicity data depend on 30 min exposure of bacteria to a treated and untreated diclofenac solution.

Results and discussion

Removal of diclofenac by fungal sources
In order to determine the most efficient fungal source for removal of diclofenac, *Yarrowia lipolytica, Phanerochaete chrysosporium, Aspergillus niger* and *Trametes trogii* cultures were incubated with 50 mg L^{-1} diclofenac for 48 h. After 48 h incubation *Aspergillus niger* and *Trametes trogii* showed 100% diclofenac removal rate. *Yarrowia lipolytica* and *Phanerochaete chrysosporium* showed lower activity, 48% and 56%, respectively (Figure 1). Although, *A. niger* (0.71 g dry weight) cultures growth rate was higher than that of *T. trogii* (0.047 g dry weight) cultures, they show the same removal capability. To examine which fungal strain was more efficient than the other, *Aspergillus niger* and *Trametes trogii* cultures were incubated with 50 mg L^{-1} diclofenac for 24 h. After 24 h incubation period *T. trogii* showed maximum removal percentage with 100% *and A. niger* showed 70% diclofenac removal percentage (Figure 2). In conclusion, *T. trogii* was chosen as the most efficient strain for this study. As depicted in Figure 3.50 mg L^{-1} diclofenac was almost completely removed (97%) from culture media in 5 hours. After 6 h incubation, diclofenac was not found in culture media. *T. trogii* is a member of white-rot fungi. White-rot fungi can degrade various aromatic compounds such as PCBs, PCP, PAHs, DDT and phenanthrene (Ryu et al. 2000). Also, *Funalia trogii* can degrade olive mill wastewater and synthetic dyes with its ligninolytic enzymes (Yesilada et al. 1995, Park et al. 2007).

A previous study showed that white-rot fungus *Trametes versicolor* completely degrade 10 mg L^{-1} diclofenac in 4 h (Marco-Urrea et al. 2010b). Hata et al. (2010) reported that *Phanerochaete sordida* completely removed diclofenac after 6 days of incubation (Hata et al. 2010). Another white-rot fungi *Phanerochaete chrysosporium* remove 93% of diclofenac after 30 days of treatment (Domaradzka et al. 2015). Esterhuizen--Londt et al. (2017) reported that aquatic fungus *Mucor hiemalis* could remove significant amount of diclofenac in 24 h

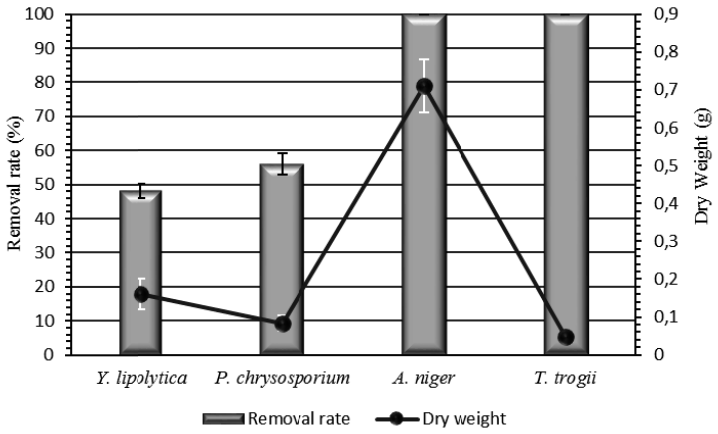

Fig. 1. Diclofenac removal capabilities of *Y. Lipolytica, P. chrysosporium, A. niger* and *T. trogii* (After 48 h incubation)

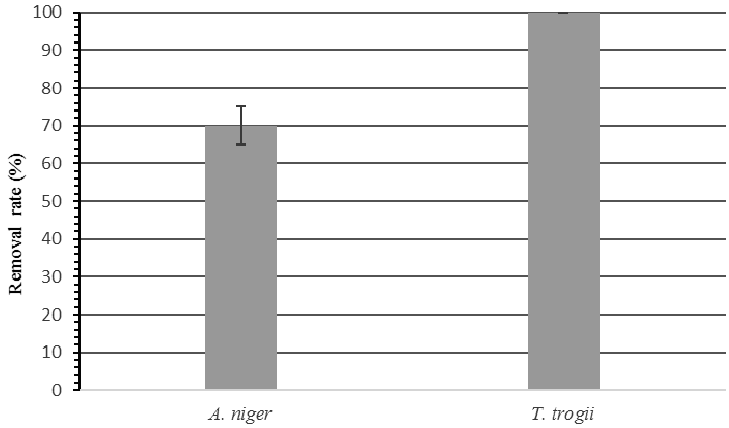

Fig. 2. Diclofenac removal capabilities of *A. niger* and *T. trogii* (After 24 h incubation)

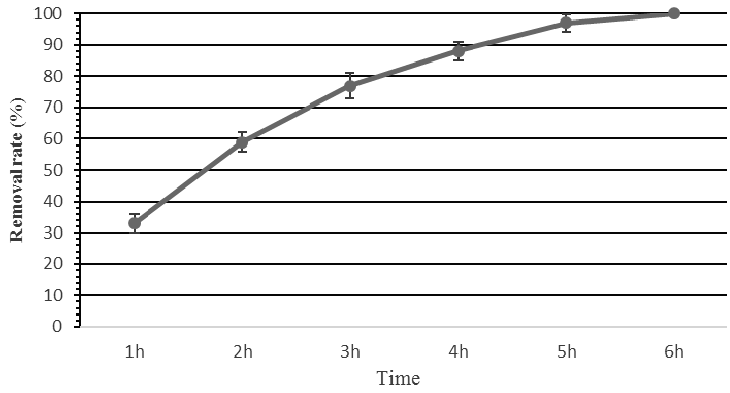

Fig. 3. Time course removal of diclofenac with *T. trogii*

(Esterhuizen-Londt et al. 2017). According to our results we suggest that *T. trogii* is a good candidate for the removal of micropollutant diclofenac.

In order to determine the role of adsorption in diclofenac removal heat killed controls (0.05 g dry weight) were used. When compared heat killed cells with living cells, heat killed cells reached only 5% removal rate. Consequently, *Trametes trogii* removes diclofenac via biodegradation rather than biosorption.

Identification of metabolites

To identify the degradation metabolites of diclofenac, LC/MS ESI (+) analyses were performed. According to LC/MS chromatogram three peaks were observed, metabolite **1** (t_R 4.77), metabolite **2** (t_R 4.93) and untransformed diclofenac (t_R 5.80) (Figure 4). Diclofenac gave a molecular major ion of [M+H] at m/z 296 (100%) (Figure 5).

Metabolite **1** and metabolite **2** exhibit the same major ion at m/z 312 [M+H] (Figures 6 and 7). Molecular ion at m/z 312

[M+H] is 16 mass unit higher than mother compound = diclofenac. The 16 mass unit increase shows hydroxylation of diclofenac moiety. Most probably, one of the aromatic rings of diclofenac was hydroxylated. In human liver, the formation of 4-hydroxydiclofenac is catalyzed by CYP2C9 enzyme (Leemann et al. 1993). Besides human liver, fungal species transform diclofenac into hydroxylated diclofenac derivatives. Fungus *Epiccocum nigrum* achieved conversion of diclofenac to of 3'-hydroxydiclofenac, 5-hydroxydiclofenac and 4-hydroxydiclofenac (Webster et al. 1998). Hata et al. (2010) identified 4-hydroxydiclofenac, 5-hydroxydiclofenac and 4,5-dihyroxydiclofenac as fungal metabolites of diclofenac (Hata et al. 2010). Marco-Urrea et al (2010) identified two hydroxylated metabolites, 4-hydroxydiclofenac and 5-hyroxydiclofenac in *Trametes versicolor* culture media. In the same study authors reported that laccase was catalyzed formation of 4-(2,6-dichlorophenylamino)--1,3-benzenedimethanol from diclofenac (Marco-Urrea et al. 2010b). In this study, we observed the formation of two hydroxylated diclofenac derivatives in white-rot fungus *Trametes trogii* culture media in 6h. Hence, based on the literature, hydroxylated metabolites of diclofenac might be 4-hydroxydiclofenac and 5-hydroxydiclofenac. After 24 h of incubation, there was no metabolite observed in culture media. Thus, *Trametes trogii* could mineralize diclofenac or transform it into undetectable metabolites.

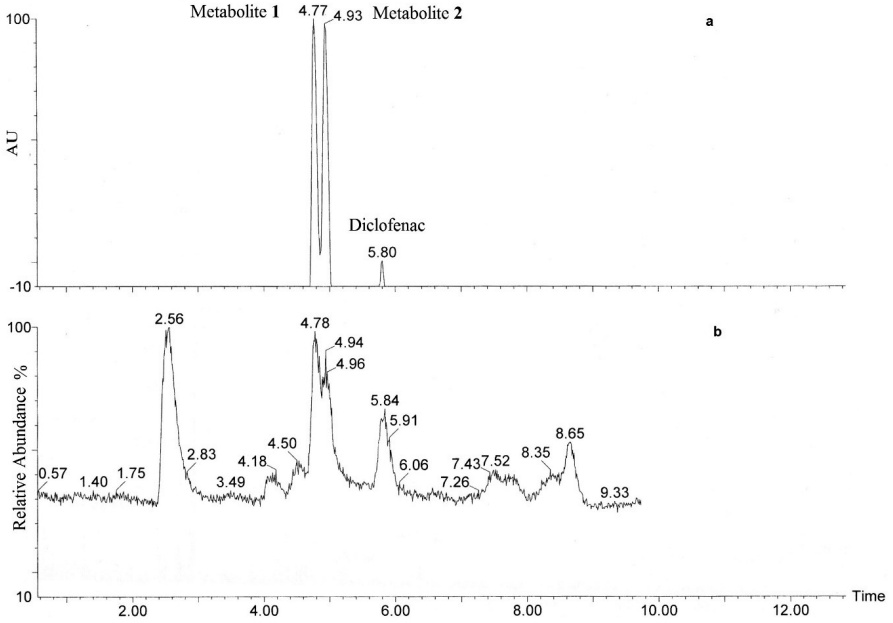

Fig. 4. LC (a) and total ion chromatogram (b) of *T. trogii* medium extract

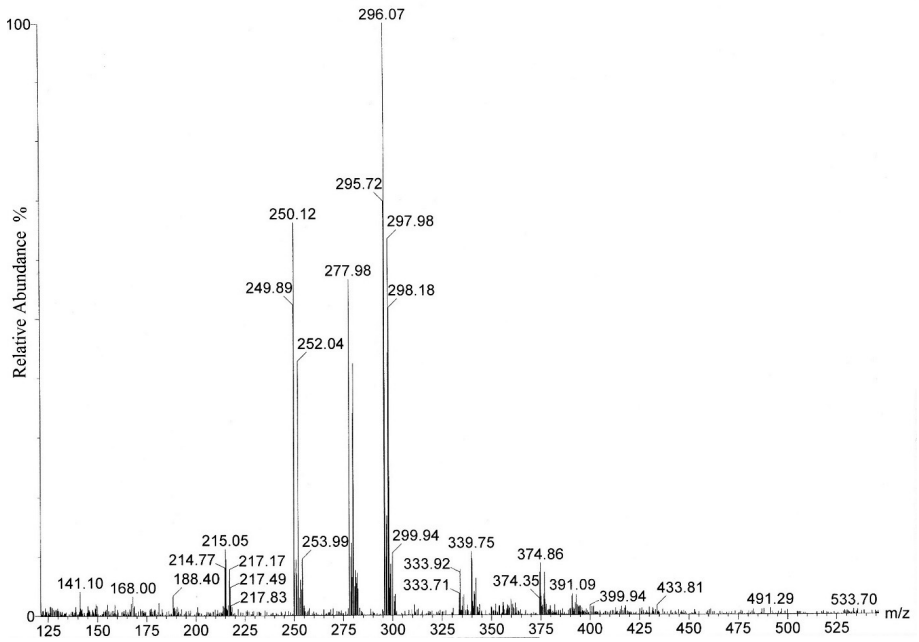

Fig. 5. Full scan ESI (+) mass spectrum for diclofenac

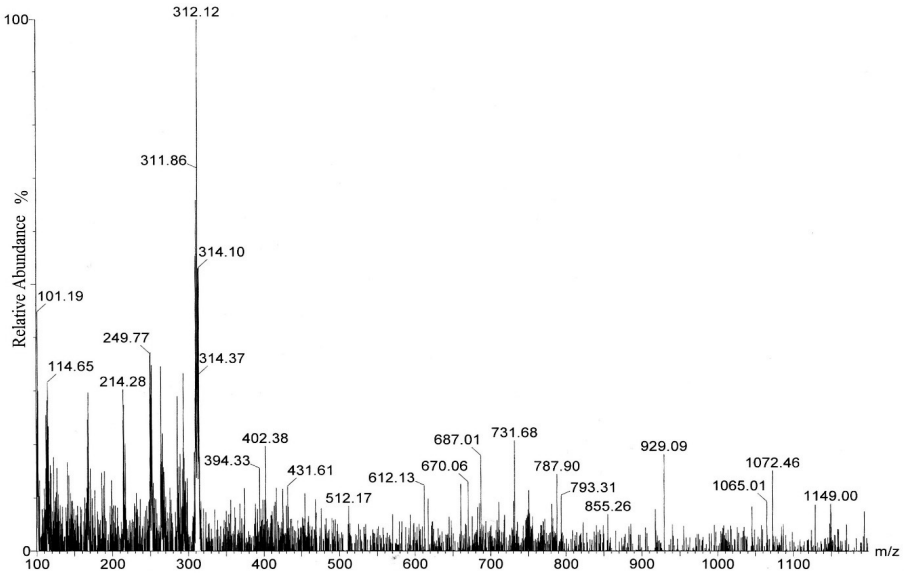

Fig. 6. Full scan ESI (+) mass spectrum for metabolite 1

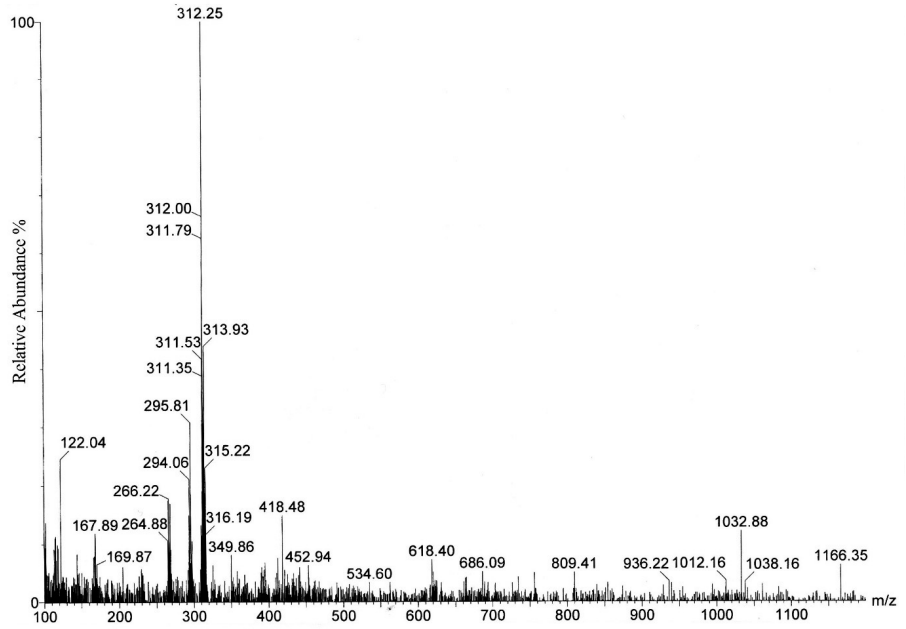

Fig. 7. Full scan ESI (+) mass spectrum for metabolite 2

Removal mechanism

In this study, we determined two hydroxylated diclofenac metabolites. In human liver, 3-hydroxydiclofenac, 4-hydroxydiclofenac, 4,5-hydroxydiclofenac and N,5--dihydroxydiclofenac are formed (Bort et al. 1999). Fungi can achieve transformation reactions such as hydroxylation of polyaromatic hydrocarbons and steroid hormones. Biotransformation reactions are often performed by monooxygenase enzymes, which belong to cytochrome P450 enzyme system (Brink et al. 1998). Fungal cytochrome P450 system performs many conversion reactions such as O-demethylation, hydroxylation, dealkylation and epoxidation. Fungal CYP504A1 enzyme performs conversion of phenylacetate to 2-hydroxyphenyl acetate (Crešnar and Petrič 2011). Marco-Urrea et al. reported that cytochrome P450 enzyme system plays a key role in diclofenac degradation with *Trametes* versicolor. In addition, they identified two hydroxy metabolites, 4-hydroxydiclofenac and 5 hydroxydiclofenac (Marco-Urrea et al. 2010b). Another study showed that *Phanerochaete sordida* transformed diclofenac to 4-hyroxydiclofenac, 5-hydroxydiclofenac and 4,5-dihydroxydiclofenac. The authors suggested that hydroxylation of diclofenac was catalyzed via cytochrome P450 system (Hata et al. 2010). Therefore, hydroxylation of diclofenac in *Trametes trogii* might be catalyzed via fungal cytochrome P450.

Culture media showed 1.8 U/ml laccase activity and 1.2 U/ml tyrosinase activity. There was no lignin peroxidase and manganese peroxidase was determined. In order to reveal the role of extracellular enzyme, 2.4 U/ml comercial laccase (pH 4.5 sodium acetate buffer) and 2 U/ml tyrosinase (pH 7 phosphate buffer) were used.

After 24 h incubation period diclofenac completely degraded in laccase containing flasks but tyrosinase did not show any effect on diclofenac (Figure 8). Also, laccase containing media turned into brownish color. Laccase, EC 1.10.3.2, p-diphenol:dioxygen oxido-reductase catalyzes oxidation of various compounds such as trichlorophenol, alkenes, industrial wastes, dyes and herbicides (Mayer 2004). A previous study showed that diclofenac was quickly transformed by commercial laccase. The authors reported that 10 $\mu g^{-1}L^{-1}$ diclofenac was transformed in 30 min by 6000 U/L laccase (Tran et al. 2010). Lloret et al. (2010), reported that diclofenac was removed by laccase. Also, the authors indicated that the presence of redox mediators syringaldehyde and 1-hydroxybenzotriazole enhanced laccase catalyzed diclofenac removal (Lloret et al. 2010). Satihskumar et al. reported that laccase mediated transformation of diclofenac (Sathishkumar et al. 2014). 4-(2,6-dichlorophenylamino)--1,3-benzenedimethanol was identified as a product of laccase catalyzed diclofenac transformation (Marco-Urrea et al. 2010b). According to our observations, we suggest that combine cytochrome P450 system and laccase play a key role in the removal of diclofenac.

Removal of diclofenac by using crude laccase

Supernatant, which showed 2.1 U/ml laccase activity, was used as a crude enzyme source. Crude laccase almost completely removed (97%) diclofenac in 48 h. In order to determine the effect of initial pH on the removal of diclofenac various pH values were tested. Maximum diclofenac removal was observed as 95% and 97% at pH 4.5 and 5, respectively. Diclofenac removal was begun to decrease more acidic or more alkali pH values (Figure 9). Laccase from *Myceliophthora thermophile* showed maximum activity at pH ranging from 3 to 4. Also, the authors indicated that diclofenac degradation strongly depends on pH changes (Lloret et al. 2010). Crude laccase from *Trametes versicolor* showed maximum activity at pH 4.5 value and the crude enzyme showed great stability at pH 5 and 5.5 (Stoilova et al. 2010). Crude laccase from *Lentinus Polychrous* showed maximum decolorization at pH 5 (Ratanapongleka and Phetsom 2014). *Funalia trogii* decolorized Reactive Black 5 effectively at pH 4.78 (Mazmanci and Unyayar 2010). Our results show that the removal of diclofenac with crude laccase prefers acidic conditions.

Nine different temperatures (15, 20, 25, 30, 35, 40, 45, 50 and 55°C) were tested at pH 5 to find the best temperature for diclofenac removal with crude laccase. Crude laccase showed the diclofenac removal below 90% in the range of 25 and 35°C in 48 h (Figure 10). Maximum diclofenac removal was observed at 30°C with 99% removal rate. A previous study reported that crude laccase from *Trametes versicolor* showed maximum activity at 45°C and the crude enzyme showed

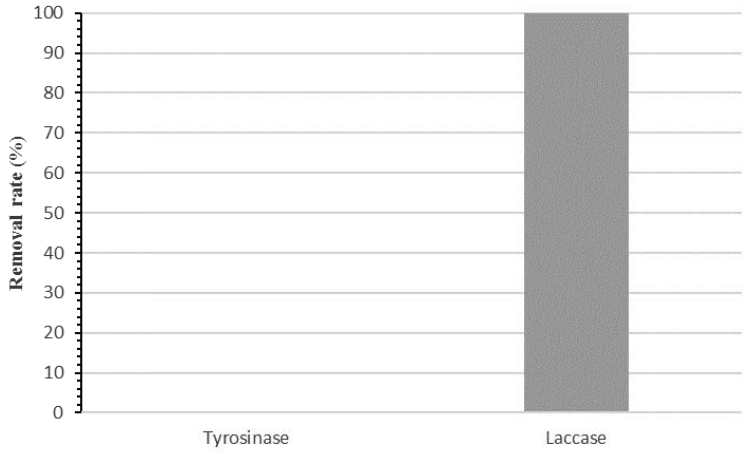

Fig. 8. Diclofenac degradation capabilities of tyrosinase and laccase enzymes

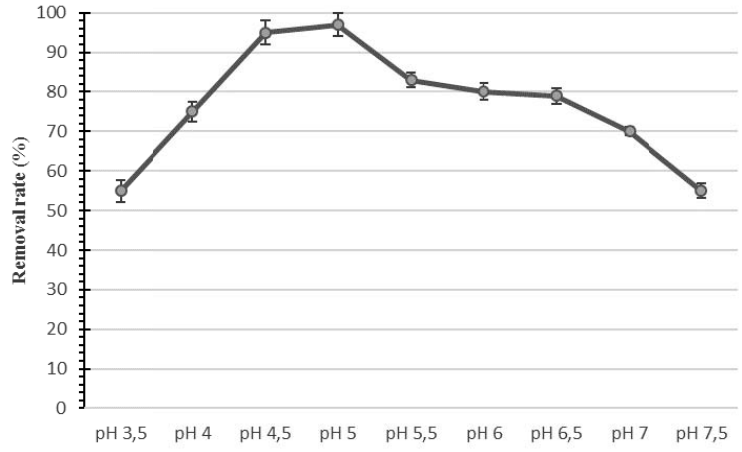

Fig. 9. Effect of pH on degradation of diclofenac with crude laccase

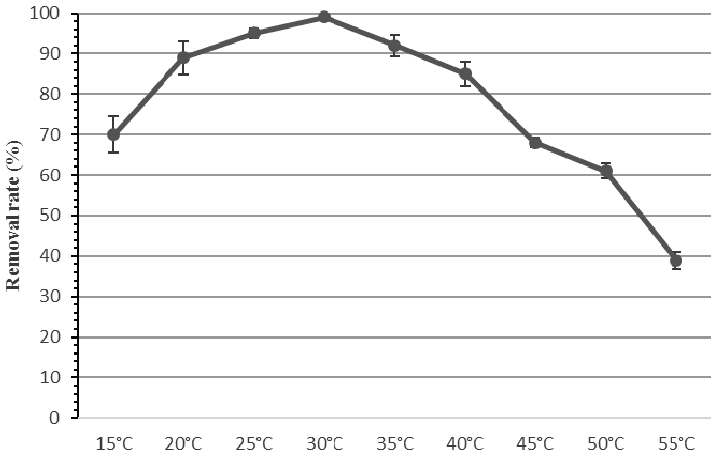

Fig. 10. Effect of temperature on degradation of diclofenac with crude laccase

greatest stability at 30°C (Stoilova et al. 2010). Culture filtrate of *Funalia trogii* showed maximum decolorization activity of Remazol Brilliant Blue R at 50°C (Deveci et al. 2004). Whereas in our study, crude laccase reached only 60% removal rate at 50°C. Another study showed that *F. trogii* showed maximum decolorization activity of at 30°C (Mazmanci and Unyayar 2010). In this study crude laccase removed diclofenac below 60% in the range of 15–50°C but removal rate reached peak 30°C with 99% removal rate.

Toxicity assay

Microtox assay was performed to evaluate changes in toxicity of diclofenac before and 24 h after treatment. Only untreated diclofenac at 50 mg L^{-1} showed a toxic effect in inhibition of luminescence with 26% toxicity in 30 minutes. Marco-Urrea et al. reported that 10 mg L^{-1} diclofenac treated with *Trametes versicolor* did not produce any toxic effects (Marco-Urrea et al. 2010b). In another study, diclofenac treated with laccase showed less toxic properties than mother compound (Sathishkumar et al. 2014). Our study showed that degradation of diclofenac with *Trametes trogii* leads to decrease in toxicity.

Conclusion

In this study, *Trametes trogii, Aspergillus niger, Yarrowia lipolytica* and *Phanerochaete chrysosporium* were tested in terms of diclofenac removal capacity. We found that *T. trogii* showed the best diclofenac removal rate with 100 percentage. Two hydroxylated diclofenac metabolites were determined in *T. trogii* culture medium by using LC/MS. Crude laccase from *T. trogii* could remove diclofenac in reaction medium.

We suggest that fungal cytochrome P450 system and laccase play a key role in diclofenac degradation. Toxicity was monitored by Microtox assay. According to Microtox assay toxicity of diclofenac was decreased when treated with *T. trogii.*

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