

Pleurotus ostreatus and *Trametes versicolor*, Fungal Strains as Remedy for Recalcitrant Pharmaceuticals Removal Current Knowledge and Future Perspectives



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Abstract

The consumption of pharmacologically active substances (PhACs) is steadily increasing. According to their recalcitrant nature and low biodegradability in wastewater treatment plants (WWTPs), they occur in rivers, lakes, and even ground and drinking waters. Because of that PhACs are considered to be emerging pollutants. Therefore new technologies which can be used to degrade them are investigated. Two white-rot fungi (WRF) strains: *Pleurotus ostreatus* and *Trametes versicolor* show quite good PhACs removal values. Broad applications of active and inactivated form of biomass are possible due to different mechanisms of their action, such as biodegradation by extracellular and intercellular enzymes or biosorption. Thus, the versatility for applying fungi, represent that this microorganisms are a promising tool to deal with problem of pharmaceutical compounds elimination from the environment. In this work has been also presented future possibilities, like their application in waste water and sludge treatment or future perspectives of removal of different group of pharmaceuticals by this promising and environmentally friendly technology

Keywords: Biosorption; Biodegradation; Fungi; Pharmaceuticals; *Pleurotus ostreatus*; *Trametes versicolor*

Abbreviations: ABTS: 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); HOBT: Hydr Oxy Benzo Triazole; MnPs: Manganese-Dependent Peroxidases; PhACs: Pharmacologically Active Substances; STPs: Sewage Treatment Plants; WRF: White-Rot Fungi; WWTPs: Waste Water Treatment Plants

Introduction

The consumption of pharmacologically active substances (PhACs) is steadily increasing, up to thousands of tons per year [1-3]. Unfortunately great amount of them cannot be easily removed through the conventional technologies used in wastewater treatment plants (WWTPs) [3-5]. Their recalcitrant nature cause their occurrence, as unmetabolized substances or as active metabolites in ng/l or µg/l in rivers, lakes, and even ground and drinking waters [1,4-6]. Because of that PhACs are considered to be emerging pollutants [1,4,6]. Even if their concentrations in water bodies are three to four orders of magnitude lower than those required producing pharmacological effects, bioaccumulation and biomagnification processes may occur [1]. The quality of aquatic environment has also the potential adverse effects on human health [7]. In addition, although risk for acute toxic effects for both fauna and flora is unlikely, chronic environmental toxic effects cannot be excluded [1,4].

Because of that new technologies which can be used to degraded pharmaceuticals are investigated [3]. Fungal treatment of wastewaters has been pointed out as a promising technology for pharmaceutical remediation processes [3,8,9]. White-rot fungi (WRF), which belong to basidiomycetes, have a vast range of the unspecific enzymatic systems, and are capable of degrading different groups of xenobiotic compounds at very low concentrations [3,4,10-12]. Besides living cells mechanism, which involves enzymes that cause biodegradation, also biosorption both on activated and inactivated biomass take part in recalcitrant pollutants elimination [8,9]. Two of the most promising fungal strains, which show quite good PhACs removal values, are *Pleurotus ostreatus* and *Trametes versicolor*. They are able to eliminate wide range of pharmaceuticals such as β-blockers (atenolol), antiepileptic (carbamazepine), analgesic (ibuprofen), anti-inflammatory (ketoprofen and naproxen) and estrogens (17α-ethynylestradiol), which suggests

the feasibility of this group of microorganisms for pharmaceuticals bioremediation purposes [5,11,13].

Pharmaceuticals Removal by *Pleurotus ostreatus* and *Trametes versicolor*

Even though elimination of many pharmaceuticals in conventional sewage treatment plants (STPs), due to their hydrophilicity, persistent nature, and the relatively low concentration is more difficult than that of other organic pollutants, white-rot fungi show great potential in removal of these chemical compounds [14]. In (Table 1) it has been set together *Pleurotus ostreatus* and *Trametes versicolor* ability to remove pharmaceuticals, with the role

of sorption and enzymatic mechanisms. While analyzing different studies it is important to keep in mind, that each experiment had its own specific methodology. Percentage of degradation could depend from medium where the trial was conducted and time of experiment. For example, *Pleurotus ostreatus* completely remove the synthetic hormone 17 α -ethynylestradiol (in concentration 200 μ g) in 3 days from a liquid complex or in 14 days from mineral medium [15]. Sometimes it could cause limitation of fungal ability to remove specific pharmaceutical. In Palli et al. [5] research atenolol degradation was negligible (<20%) during the first 20 days but it increased up to 60% after notable biomass growth (130% when compared to the initial inoculum) of *P. ostreatus*.

Table 1: *Pleurotus ostreatus*, *Trametes versicolor* and their enzymes ability to remove pharmaceuticals.

pharmaceutical	Fungal Strain				Enzyme		
	Pleurotus ostreatus		Trametes Versicolor		laccase	Cytochrome P450	MnPs
	Biodegradation	Sorption	Biodegradation	Sorption			
10,11-epoxycarbamazepine	-	-	100 ^[23]	-	-	-	-
17 α -ethynylestradiol	100 ^[7,15]	19 ^[7]	-	-	97.100 ^[8,16]	-	>99 ^[16]
17b-estradiol 17-acetate	-	-	>80 ^[18]	>80 ^[18]	-	-	-
Acetaminophen	-	-	100 ^[23]	-	-	-	-
Acridon	0-60 ^[5]	-	100 ^[23]	-	-	-	-
Atenolol	68-100 ^[4,16]	-	-	-	100 ^[5]	0 ^[5]	-
Carbamazepine	-	-	<30-98 ^[3,6,14,29]	6-17 ^[3,9,14]	5-37 ^[14,16]	p.r. ^[26]	14.20 ^[16]
Ciprofloxacin	55 ^[4]	-	>90 ^[21]	-	16 ^[21]	p.r. ^[21]	-
Clarithromycin	-	-	-	-	-	-	-
Clofelic acid	-	-	<30-97 ^[14,18,29]	12.5 ^[14]	7-20 ^[14,16]	p.r. ^[29]	<10 ^[16]
Codiene	-	-	100 ^[23]	-	-	-	-
Citalopram	100 ^[5]	30 ^[5]	100 ^[23]	-	-	-	-
Diclofenac	-	-	54-100 ^[3,6,14]	10-80 ^[3,5,6,14]	90-100 ^[5,14,16]	p.r. ^[5,14]	100 ^[16]
Erythromycin	-	-	100 ^[23]	-	-	-	-
Fenoprofen	-	-	100 ^[14]	7 ^[14]	20 ^[14]	-	p.r. ^[16]
gemfibrozil	50 ^[5]	-	83 ^[14]	17 ^[14]	20-30 ^[14,16]	p.r. ^[18]	30 ^[16]
Ketoprofen	-	-	<30-100 ^[14,17,18]	0-15 ^[5,14,17]	0-50 ^[5,14,18]	p.r. ^[5,17]	22 ^[16]
Iopromide	-	-	39-46 ^[3]	1-8 ^[3]	-	-	-
Ibuprofen	-	-	100 ^[4,14,29]	17 ^[14]	<5-40 ^[14,16,18]	p.r. ^[18]	20 ^[16]
Indomethacin	-	-	100 ^[14]	15 ^[14]	>90 ^[14]	-	p.r. ^[16]
Metonidazol	-	-	100 ^[23]	-	-	-	-
Naproxen	-	-	31-100 ^[13,14,22]	12.5-23.7 ^[14,22]	10-100 ^[16,22]	100 ^[22]	95 ^[16]
Norfloxacin	-	-	>90 ^[21]	-	16.3 ^[21]	p.r. ^[21]	-
Prophenazine	-	-	64 ^[14]	10 ^[14]	7 ^[14]	-	-
Sulfamethazine	-	-	>95 ^[27]	25 ^[27]	22 ^[27]	p.r. ^[27]	-
Sulfapyridine	-	-	100 ^[19]	neg. ^[19]	75-98 ^[19]	-	-
Sulfathiazole	-	-	100 ^[19]	17 ^[19]	82-100 ^[19]	p.r. ^[19]	-
Venlafaxine	-	-	49-53 ^[3]	2-6 ^[3]	-	-	-

Note: p. r. - potential role, neg. - negligible role, no data

So atenolol is degraded by the fungus, but it requires a long contact time and high levels of biomass production [5]. As mentioned above biomass, but also pharmaceutical concentration is not without significance. As an example 94% of carbamazepine

with initial concentration 9mg/L have been removed when treated by *T. versicolor* for 6 days, but only 61% of the contaminant was degraded, when initial amount was 50 mg/L [9]. It sometimes cause difficulties in comparing result from different researches,

like those above with 68% removing of carbamazepine in 7 days treated with *P. ostreatus*, when the input value was 4 µg/L [4]. Interestingly even the method of result evaluation has the influence on the outcome. It was found 64% of degradation of diclofenac treated with *T. versicolor* when the reduction was measured directly (with pharmaceutical concentration in both liquid and biomass), and only 54% when was measured indirectly (by subtracting the degradation value measured in the liquid from the killed control culture from the degradation measured in the liquid from the experimental culture from the batch experiments with fungi and spiked synthetic medium) [3].

Role of Sorption

Removal mechanisms during treatment with WRF include: sorption on the fungal biomass, degradation by extracellular enzymes, and degradation by mycelium bound or intercellular enzymes [16]. In case of pharmaceuticals overall elimination by fungi the contribution of sorption, fast, reversible and energy-independent process, cannot be neglected [3,17]. It gathers both absorption (entry of pollutants inside the biomass) and adsorption (adhesion of pollutants to the biomass surface). It depends on fungus, because specific interactions between PhACs and the surface components of each fungus can occur. The differences also take place, while sorption is on the active or inactive (killed) biomass [3]. Structure of biomass, and therefore their sorption capacities, may change according to the inactivation mechanism [3,11]. In active

biomass, transport in living cells may play an important role [3,8]. In addition, biodegradation processes of absorbed compounds can occur in the active biomass due to intracellular enzymes [3]. On the other hand metabolically active biomass, may suppressed toxic pollutants by cellular protective mechanisms [8].

What is more, way of biomass inactivation can have influence on sorption. These presents Palli et al. [5] research, where heat-killed biomass of *T. versicolor* remove 47% of diclofenac and 15% of ketoprofen, but these percentages were reduced to 10% and 0%, respectively, when sodium azide was used to inactivate biomass, by blocking active transport across membrane or vesicular pathways [5]. Not without significance is chemical character of pollutants. In Nguyen et al. [18] it has been reported high (60-99%) removal of hydrophobic compounds, to which belongs naproxen or ibuprofen by the live culture, but relatively low by the inactivated one, which indicates biodegradation was the main mechanism. Hydrophilic one on the other hand showed negligible removal by both active and inactivated culture, which may indicate the importance of sorption in subsequent degradation by the whole-cell [18]. The biosorption mechanisms are classified to different types on the basis of cell metabolism status or pollutants sorption location [8], and have been presented at (Figure 1). The drawback of sorption is that fungal biomass after treatment might be considered as a potential waste, which requires appropriate treatment before being released into the environment [3].

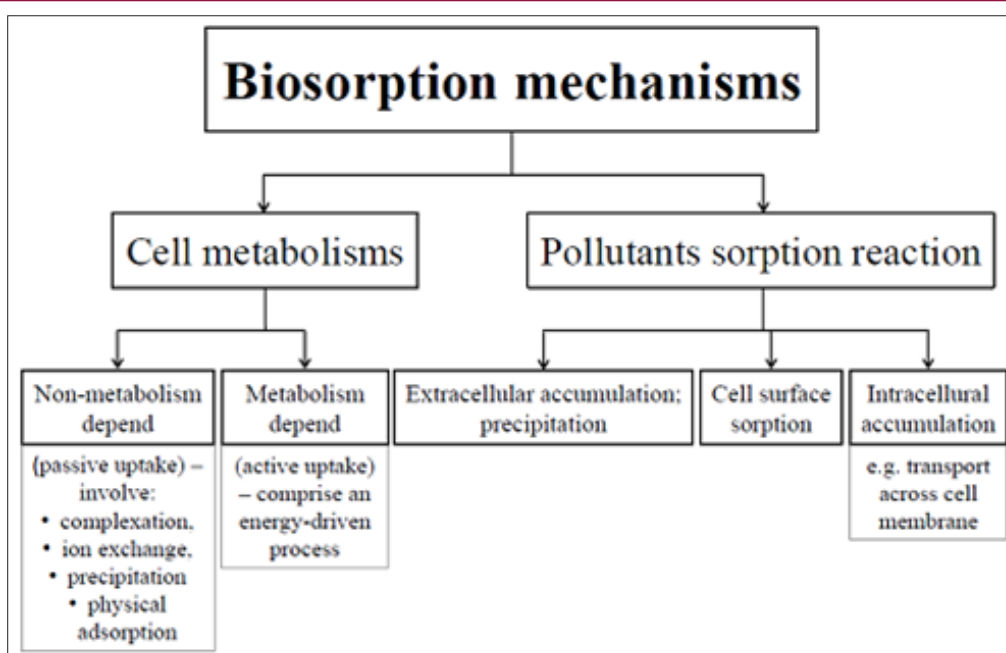


Figure 1: The biosorption mechanisms classified to different types on the basis of cell metabolism status or pollutants sorption location after Lu.

Enzymes Contribution in Pharmaceuticals Degradation

Biodegradation by whole-cell can be due to intracellular (cytochrome P450), extracellular (laccase, manganese-dependent peroxidases - MnPs), mycelium-associated enzymes and their synergetic effects [1,5,16,18,19]. This can lead to significant differences in removal by whole cell WRF and harvested enzyme. For example carbamazepine removal by *P. ostreatus* is 100%, by

T. versicolor 98%, whereas crude and purified laccase could only achieve 5-37% removal. Similarly, ibuprofen was completely removed by whole-cell WRF, while its removal by crude and purified laccase was in the range of 5-40%. It substantiates the role of mycelium bound and/or intercellular enzymes [16]. Indeed, the role of cytochrome P450 in the degradation of naproxen, diclofenac, and carbamazepine has been demonstrated in studies where those

pharmaceuticals were only partially removed (15-50%) in the presence of cytochrome P450 inhibitor [9,11,16,20].

What is more there are significant differences depending on the origin of the enzymes. Almost complete removal of three pharmaceuticals: diclofenac, ibuprofen, and naproxen, have been achieved after treatment with crude enzyme extracted from *T. versicolor*, whereas purified laccase from this fungi strain achieved only 20–50% removal [16]. To similar conclusions on the efficacy dependence from the origin of the enzyme have come Tran et al. [14] with researches with *T. versicolor*, cell-free extract (crude laccase), and commercial laccase, examined on diclofenac and naproxen [14]. On the other hand purified laccases are more effective for the removal of phenolic compounds and usually ranges from 70 to 99%. Even though that for most non-phenolic compounds removal by crude enzyme is <20%, it's worth to mention that relatively higher removal of diclofenac (40-50%), and ibuprofen (30-45%), has been reported because these compounds contain both electron donating and electron withdrawing functional groups [16].

Not without significance is also enzyme activity, like in Nguyen et al. [18] work, where when laccase activity increase from 2 to 6 U/mL, reported removal of naproxen changed from 30 to 100%, ibuprofen from 10 to 40% and from 25 to 50% in case of ketoprofen [18]. What is more, sometimes there is a need for mediator, like in Prieto et al. [21] researches, when in in vitro assays with purified laccase after adding enzyme mediator ABTS - 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, norfloxacin and ciprofloxacin removal increased from 0% to 33.7% and from 16.3% to 97.7% respectively [21]. Also carbamazepine degradation by purified laccase increased from 0% to 60% after addition of a redox mediator HOBt (hydroxybenzotriazole) in Jelic et al. [9] work or naproxen from 10% to 95%, what was presented in Marco-Urrea et al. [11,13,17,20,22,23] article [9,22,23].

Current and Future Possibilities of *Pleurotus ostreatus* and *Trametes versicolor* Use

Pleurotus ostreatus and *Trametes versicolor* use for removing pharmaceuticals is one of the most promising bioremediation systems, because these WRF are well known as producers of non-specific enzymes, which allow to simultaneous attack complex mixtures of pollutants [1,9,22]. In addition preconditioning of fungal strains to particular pollutants is not require, because part of the enzymes useful in the degradation are produced constitutively while others after induction by nutrient limitation [1,22]. Because of that they could find application in wastewater and sludge treatment.

Waste Water Treatment Application

There have been conducted several studies about *P. ostreatus* and *T. versicolor* wastewater treatment application [5]. Unfortunately, researches presented by Cruz-Morató et al. [23] showed that in fluidized bed bioreactor inoculated with *T. versicolor* biomass used for urban wastewater treatment in non-sterile conditions pharmaceuticals removal can change. When comparing with the removal in sterile conditions some drugs elimination such

as acetaminophen, ibuprofen, citalopram was the same (100%), some like ketoprofen increased (from 35% in sterile to 100% in non-sterile), and some, like in case of 10,11-epoxycarbamazepine decreased (from 100% in sterilized urban wastewater to 79% in non-sterilized) [23]. Also Yang et al. [24] proved that diclofenac removal by *T. versicolor* in membrane bioreactor (MBR) efficiency decrease from 100% in sterile conditions to 55% in non-sterile [24]. Even though white-rot fungi capacity to successful degradation of pharmaceuticals is irrefutable the problems while application in real urban wastewater occur. They are: continuous fungal and their extracellular enzymes washout in continuous flow reactors, contaminants mixtures, PhACs low concentrations and mentioned before non-sterile environment [23,24]. Bacterial contamination affects adversely on removal efficiency, because of: competition between bacteria and fungi for substrate; fungal mycelium damaging, biomass growth disrupting and destabilization of fungal activity [16, 24]. Some of the strategies to avoid bacterial contamination are:

- Coagulation-flocculation pretreatment of wastewater, which will reduce the initial bacterial count;
- Coupling bioreactor with micro-screen, which would retain fungal biomass but allow the washout of bacteria with effluent;
- Immobilization of fungal strains onto different carriers;
- Operation under acidic pH, according to fact that optimum pH for the growth fungi is lower than that preferable for most of bacteria;
- Periodic fungi biomass replacement;
- Use of disinfecting agents (like ozone), which will selectively inactivate bacteria but will not impose any harmful effects on fungal biomass.

In case of enzymatic reactors where enzymes instead of whole-cell WRFs are used, limitation are washout of the enzyme and mediators. To avoid it could be use:

- Coupling of enzymatic reactor with a membrane with suitable pore size;
- Enzymes immobilization [16].

These possibilities enable the implementation of technology using the WRF, and minimizing inconvenience. It is worth to mention, that had been proved that, besides pharmaceuticals removal, *P. ostreatus* is able to reduce chemical oxygen demand of the hospital wastewater which is an important advantage [5].

Sludge Treatment

WRF might be applied not only at wastewater treatment plant itself, but also in elimination pharmaceuticals form sludge. Bio solids may still contain significant amounts of drugs and currently the development of alternative strategies for its treatment is a matter of concern [10,11]. Fungi are robust organisms and are more tolerant to high concentrations of contaminants than bacteria [10]. The

Rodríguez-Rodríguez et al. [13,19,26] researches results suggest that a fungal treatment with *T. versicolor* could be a promising process [11,13]. His another researches (2011) of pharmaceuticals biodegradation by this fungi in sterilized sewage sludge under solid-phase conditions demonstrate complete removal of phenazone, bezafibrate, fenofibrate, cimetidine, clarithromycin, sulfamethazine and atenolol after treatment, while seven others pharmaceuticals were removed between 42% and 80% [25]. In his another work (2012) presented *T. versicolor* in a sludge-bioslurry reactor, when fifteen out of 24 detected pharmaceuticals were removed at efficiencies over 50% after the treatment (such as diclofenac, ibuprofen, indomethacine, sulfamethazine, sulfathiazole), including eight completely degraded (e. g. sulfapyridine) [26].

What is more García-Galán et al. [27] demonstrated degradation capacity of *T. versicolor* in sterilized sewage sludge, where 100% removal was accomplished for sulfapyridine and sulfathiazole [27]. Also Palli et al. [5] evidenced carbamazepine degradation by *P. ostreatus* in solid state fermentation [5]. In addition both Aydin [10] and Rodríguez-Rodríguez et al. [25,27] researches

showed significant reduction in toxicity of sludge, which contain pharmaceuticals after treatment with *T. versicolor* [10,25]. It all demonstrating the potential application of the fungus for sewage sludge bioremediation [27,28].

Future Possibilities

On the base of presented knowledge have been tested tolerance of *Pleurotus ostreatus* and *Trametes versicolor* to anticancer drug - bleomycin. Pure cultures of selected microorganisms were isolated with tissue method from fruit bodies and propagated. Their ability to grow in the presence of selected substance, were conducted by placing 8 mm fungi disc on solid media (Malt Extract Agar) with addition of two different concentrations of chosen cytostatic (1mg/L and 4mg/L) and incubating for five days in 26°C. Test have been conducted in five repetitions. Their tolerance was designated by growth ability presented in cm (diameter of colony), which show (Figure 2). Even though, growth inhibition is noticeable, both fungal strains are able to growth in quiet high bleomycin concentrations, which may induce that those strains will find use also in removing this cytostatic drug, but further researches are require.

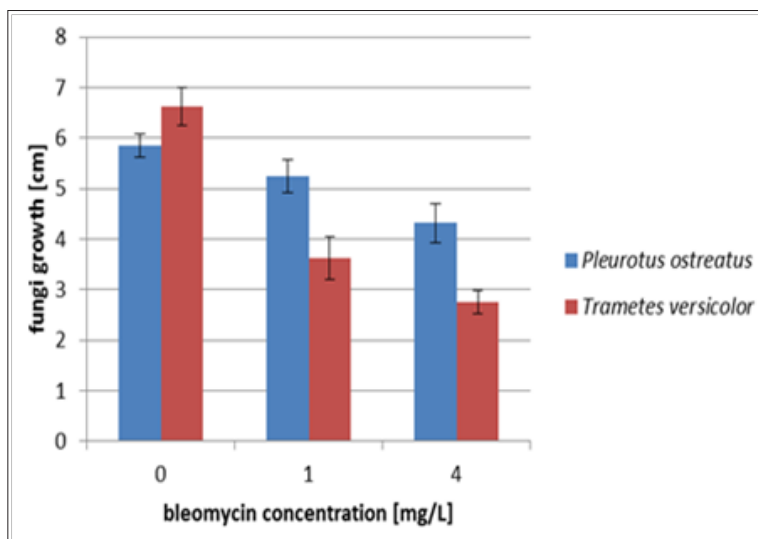


Figure 2: *Pleurotus ostreatus* and *Trametes versicolor* growth in the presence of bleomycin in concentration 1 and 4 mg/L.

Conclusion

Using white-rot fungi: *Pleurotus ostreatus* and *Trametes versicolor* in pharmaceuticals removal is a promising and environmentally friendly technology. Broad applications of both active and inactivated form are possible due to different mechanisms of their action, such as biodegradation by extracellular and intercellular enzymes or biosorption. In addition there is also a possibility of using only their extracellular enzymes. Thus, the versatility for applying fungi, represent that this microorganisms are a promising tool to deal with problem of pharmaceutical compounds elimination from the environment.

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