Removal of phenols-like substances in pharmaceutical wastewater with fungal bioreactors by adding *Trametes versicolor*

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**ABSTRACT**

Fungi are known to be more resistant to toxic compounds and more effective in removing recalcitrant organics such as phenols than bacteria. Here we examined the removal of phenols (as a component of Zopiclone drugs), added to non-sterile pharmaceutical wastewater with continuous treatment fungal bioreactor by its augmentation with mono-species of white-rot fungi (*WRF*) *Trametes versicolor*. Results showed that WRF in a sterile reactor (a batch mode) were moderately effective for removal of phenols (40% in seven days); however, native wastewater microbes at optimal conditions for fungi (pH 5.5, 25 °C) were more effective (90%, both in batch and continuous flow modes). In continuous flow mode, addition of WRF was an effective way to mitigate high loads of phenols (up to 400 mg/L), by both fungal enzymes (growth rate 0.075 h⁻¹, laccase enzymatic activity 4 nkat/mL) and biosorption. The study confirmed that naturally occurring fungi in combination with fungus-augmentation is an effective approach for treatment of high-strength pharmaceutical wastewater.

**Key words** | fungal bioreactor, pharmaceutical wastewater, phenol removal, *Trametes versicolor*

**MAIN FINDINGS**

- High concentrations of phenols were effectively removed from non-sterile pharmaceutical wastewater in batch by fungi in a week.
- Addition of white-rot fungi to a fungal reactor was an effective way to maintain high removal efficacy even during high organic loads of pharmaceutical wastewater.
- Fungi removed phenols both with enzymes and biosorption.
- Maximum specific growth rate of white-rot fungi was 0.075 h⁻¹.

**INTRODUCTION**

Due to the manufacturing process of drugs, pharmaceutical wastewater treatment plants receive a wide variety of chemicals, often with sporadic high loads of recalcitrant or toxic organic compounds (Gadipelly *et al.* 2014). Buffer tanks are usually used to dilute and equalize flows before biological treatment because bacteria are not effective for high loads of such organics (Sharda *et al.* 2013). It may lead to disfunction of water treatment plants and thus to risk of environment pollution with pharmaceutical compounds. Physico-chemical methods such as electro-coagulation, photodecomposition, membrane filtration, advanced oxidation process or adsorption could be employed for removal of recalcitrant compounds (Villegas *et al.* 2016), however, they are more expensive than biological methods, and generates by products and chemical sludge.

Fungi are more resistant to toxic compounds due to chitin-rich cell wall and both specific and nonspecific extracellular enzyme production. They can degrade diverse contaminants without long time of adaption (Sankaran *et al.* 2010). Previous studies (Bernats & Juhna 2015; Lucas *et al.* 2016) have shown that fungi could be used to treat industrial wastewater including a high concentration of phenols containing pharmaceuticals. However, applicability of fungi for (pre)treatment of high and variable loads of recalcitrant and toxic compounds, which is important of pharma factories, has not been described in literature, although there are some plants (e.g. in Sweden) which...
already are using fungi for pharma wastewater treatment. Fungi produce multiple enzymes, such as laccase and peroxidases (Lacina et al. 2003) that are able to degrade compounds with cyclic structures such as phenols or lignin (a heterogeneous polyphenolic polymer). Fungi also have excellent biosorbent properties (Bayramoglu et al. 2007), and combination of fungal metabolic and sorptive properties can be used for pharmaceutical wastewater treatment with variable contaminations.

The aim of this study was to determine the potential of fungi for removal of periodically high loads of phenols from high-strength pharmaceutical wastewater. We studied real pharmaceutical wastewater, with added high loads of phenols (as a model for recalcitrant and toxic chemicals), treatment using fungi. We examined the process in continuous flow mode by adding (fungus-augmentation) white rot fungi (WRF). Experiments of total phenol (TP) removal were done in bench-scale reactor, in both batch and continuous flow (HRT = 24 h) modes.

**MATERIALS AND METHODS**

**Synthetic wastewater and wastewater-based medium**

Synthetic wastewater (SW) was prepared from medium consisting of KH₂PO₄ 0.80 g/L, K₂HPO₄ 0.20 g/L, MgSO₄ 0.50 g/L, yeast extract 5.00 g/L, NH₄NO₃ 3.00 g/L. Chemical oxygen demand (COD) was set as c.a 20 g/L and final TP concentration – 400 mg/L. TP was prepared from a stock solution of 9.8 g/L, from the same pharmaceutical company where wastewater was obtained.

SW was autoclaved at 121 °C for 15 min, before that pH was corrected to 5.5 with 1 M HCl. Reactors also were autoclaved at the same conditions, separately, packed in aluminium folium. The medium was filled to the reactor at the fume hood, having been disinfected prior by UV light for 30 min, and sealed.

Wastewater was taken from the inflow of the pharmaceutical production company’s wastewater treatment plant and had following content: COD, 3,400 mg/L, total nitrogen (Ntot), 78 mg/L, total phosphorus, 1.6 mg/L, TP, and 12.5 mg/L. The final concentration of TP was adjusted using previously described stock solution, with a concentration of 9.8 g/L of TP.

**Fungus inoculation**

For fungus-augmentation, monoculture of basidiomycete Trametes versicolor was used. Stock culture of T. versicolor was obtained from fungal species storage of Umea University (Sweden). Prior to the stock cultures’ inoculation in agar medium, cultures were kept on sterile agar plates in the refrigerator at 5 °C. Then, in reactor experiments, stock culture from agar plate was inoculated in liquid medium of 150 mL, with media B (KH₂PO₄ 0.80 g/L, K₂HPO₄ 0.20 g/L, MgSO₄ 0.50 g/L, yeast extract 5.00 g/L, NH₄NO₃ 3.00 g/L, dextrose 3.00 g/L, pH = 5.5) and filled-up in 250 mL flasks. Correction of medium pH was done using 1 M HCl, and it was set to pH 5.0. Afterwards, flasks with medium were covered with cotton corks, and autoclaved at 121 °C for 15 min. Stock culture inoculation to flasks was done in a fume hood, which was disinfected by UV light for 30 min. After inoculation, flasks were placed in a thermostat box with orbital shaker, at 30 °C and 150 rpm for seven days. Afterwards, flask medium was homogenized with autoclaved glass spheres (2 mm in diameter), and added to reactor content previously disinfected by UV light for 30 min in a fume hood.

**Bioreactor setup**

All connections of reactor had screws with autoclave proof rubber brackets. Reactor mixing was done through non-contact electromagnetic connection between vertical mechanical shaft and mixing plates with medium. Operating volume of reactor was set to 3.12 L. Bench-scale bioreactor had the following condition controls: pH control with NaOH and HCl, temperature control with inner heat exchanger, mixing control, and aeration control. Temperature, pH and aeration controls were done with on-line probes linked with a programmable logic controller control unit, which controlled certain parameter set point. Technological scheme of bioreactor and condition control is shown in Figure 1.

**Experimental procedure**

Bioreactor experiments for TP removal efficiency estimation with T. versicolor, were divided in to two sets. First two sets were done in batch or ‘no-flow’ mode, for hydraulic retention time (HRT) of seven days. In the first experiment, removal of TP was tested with synthetic medium. The scope of this experiment was to evaluated removal efficiency at idealized conditions, with no bacterial inhibition for fungal biomass growth and overall TP removal trend observation. Batch mode experiments consisted from sterile wastewater medium supplemented with TP stock medium of 9.8 g/L to achieve necessary final concentration.

Before the start of an experiment with synthetic wastewater medium, the reactor was filled with a volume of...
3.12 L, with the following content: 2.7 L of synthetic medium, 0.12 L of phenol concentrate and 0.3 L (10%) of fungal inoculant biomass. Current fungal biomass concentration of 10% was assumed to be optimal from previous batch studies (Bernats & Juhna 2015). All content was prepared in a disinfected (UV light, 30 min) fume hood.

The first experiment involved a batch mode operation with a synthetic medium for seven days. Temperature was set to 25°C to achieve the highest phenol degradation rate in wastewater (Bernats & Juhna 2015). A medium pH was monitored through a computer programme that detected changes and compensated a departure by the addition of 1 M HCl or 5 M NaCl solutions. Mixing of reactor medium was done with non-contact gearing steering plates, with rotation speed of 150 rpm. The solution was aerated with compressed air set at 1.0 bar, at an air flow of 2.5 L/min. Evaporation was controlled by a compensator mounted on top of the reactor.

The second set of experiments were expanded in continuous flow mode, by first seven days operating in batch mode, afterwards setting flow conditions to continuous flow. In continuous flow mode, peristaltic pumps lifted wastewater from a 20 L wastewater tank to the reactor. Meanwhile, after treatment wastewater was collected in second 20 L tank, at bioreactor outflow. Flow rate in continuous mode was set to 125 mL/hour, which ensures 24 h HRT.

**Water samples and analysis methods**

All samples were taken by syringe and filled in sterile 50 mL containers which, if not analysed immediately, were placed in a fridge or freezer. The following parameters were measured in filtered (f) or non-filtered (non-f) format: TP (f, mg/L), COD (f, mg/L), biological oxygen demand (BOD) (non-f, mg/L), total organic carbon (TOC) (non-f, g/L), Biomass (non-f, relative %), optical density (OD) (non-f, mg/L) and enzymatic activity (non-f, nkat/mL). For filtered samples, 0.45 μm Minisart, high flow syringe filters were used, excluding COD and biomass measurements where 1.6 μm Whatman GF/A filters were used.

TP was measured by spectrophotometer Hach DR 5000 (HACH LANGE, Manchester, UK), with automatized
cuvette tests. For COD determination spectrophotometer Hach DR 5000 (HACH LANGE) was used, with automated cuvette tests LCK014 and LCK514, before measurements samples were re-filtered through 1.6 μm Whatman GF/A filters. For BOD determination, standard five-day BOD test with measuring dissolved oxygen was used. For TOC determination, TOC analyser 5000 A TOC analyser was used; TOC samples were collected in freezer before analysing. For biomass samples, directly after sampling, 10% formaldehyde was added and collected in fridge temperature; afterwards, before microscope analyses, samples were re-filtered through 1.6 μm Whatman GF/A filters, contacted with formaldehyde and collared with TetOn dye. For fungal biomass fluorescence, calcofluor white dye was used. For both biomass analyses, microscopes Leica DMLB (equipped with Nikon Eclipse) and Q IMAGING (equipped with Retinga 2000R) were used. OD was determined using spectrophotometer Camspec M501, at 600 nm. Enzymatic activity samples were measured immediately after sampling with a spectrophotometer.

**Fungal growth rate determination**

The intrinsic growth rate was estimated by measurements of TP and biomass concentrations. These measurements were made from solution collected from the bioreactor operating in batch mode in non-sterile conditions. Kinetic parameters of maximum specific growth rate (μmax) and half-saturation coefficient (Ks) were expressed using linearized Monod equation: \( \frac{1}{\mu} = \frac{1}{\mu_m} + (\frac{K_s}{\mu_m})\frac{1}{S} \), where \( \mu \) = specific growth rate (1/h), \( S \) = TP concentration (mg/L), \( K_s \) = half-saturation coefficient (g/L), \( X \) = fungal biomass concentration (g/L), \( X_0 \) = initial fungal biomass concentration (g/L), and \( \mu_m \) = maximum specific growth rate (1/h).

**RESULTS AND DISCUSSION**

The fungal treatment of effluents containing organic pollutants including phenols is a feasible alternative. A bold feature of this enzymatic machinery is its non-specificity, due to its action via the generation of radicals. This property makes the extracellular WRF enzymes capable of transforming a wide range of organic molecules including micropollutant (low concentration, ng/L) and industry (high concentration, g/L). However, there are several challenges in order to advance the technology towards industrial scale. The competition of fungi with autochthonous bacteria is one of the most important problems. One of the methods to overcome this problem is a periodic or continuous addition of fungi in the treatment reactors, or so called fungus-augmentation.

This study started with testing in a batch reactor removal of high concentration of phenols in sterile synthetic wastewater with bioaugmenting it using WRF *T. versicolor*. About 40% of phenols (with starting concentration of 400 mg/L) were removed during the first days, after which it decreased by 10% during the week (Figure 2). Because wastewater contained a high concentration of organic matter present in the wastewater, it appears that WRF were using more ready available organic matter as COD concentration in the sample as it decreased from 20 to 15 g/L (not shown). The removal rate of TP was within the range reported by others studies, ranging from 20% to 75% removal (Roussos et al., 2018).

**Figure 2** Degradation of TP in sterile conditions with addition *T. versicolor* stock culture (initial concentration 400 mg/L, 10%, T = 25 °C, pH = 5.5, Steering = 150 rpm, repetitions n = 2): Batch mode.


et al. 2010). Then we switched the reactor from sterile to non-sterile conditions and pH was lowered to 5.5 to discourage bacterial growth and fungus augmentation was continued. Removal efficacy increased during one week reaching 90% (Figure 3). This could be explained by metabolic activities of fungi and perhaps its synergy with bacteria, which were present in a lower concentration due to low pH value. This shows that complete phenol removal can be achieved by use of multi-cultures rather than with presence of only one monoculture (T. versicolor). Environmental parameters in non-sterile condition experiments were optimal for fungal biomass growth and limiting for bacteria biomass. General limiting parameter was pH with a value of 5.5, which with the rise of acidity reduced bacterial growth and activity, in the same time, accelerating fungal biomass growth and dominance in a fungal bioreactor. From non-sterile conditions, experiments we concluded that phenols removal can be effectively achieved also for real wastewater at sufficient adaptation time.

Final reactor experiments were extended for continuous flow mode. These experiments consisted from batch stage, with biomass adaption time for seven days (HRT = 168 h), and continuous flow stage followed afterwards (HRT = 24 h). Continuous flow experiments, analogous to non-sterile batch experiments, were done with two TP concentrations, 100 mg/L and 200 mg/L (Figure 4), and initial COD concentration in the range of 6 to 10 g/L. The results showed that phenols concentration where reduced from 100 to 2 mg/L also in non-sterile conditions at continuous flow with contact time of 24 h.

![Figure 3](image1.png)  
**Figure 3** | Degradation of TP with T. versicolor in non-sterile conditions, with addition of T. versicolor stock culture (10%, T – 25 °C, pH – 5.5, Steering – 150 rpm): Batch mode.

![Figure 4](image2.png)  
**Figure 4** | Degradation of TP with T. versicolor in non-sterile conditions (Xinitial – 10%, T – 25 °C, pH – 5.5, Steering – 150 rpm): Continuous flow mode.
Continuous treatment showed that phenols were removed because of oxidation (correlation with enzymatic activity and washout rates), however, bioaugmentation with WRF allows the increase of efficacy. Here we propose that for accommodating high sporadic loads of pharma, wastewater continuous treatment with bioaugmentation could be a viable option.

Fungi might be used for removal of recalcitrant compounds, including phenols, because of non-specific and non-selective enzyme systems, that enable them to degrade complex individual and mixtures of pollutants. Thus, application of fungi could also be used as the pre-treatment step.

To evaluate the importance of biosorption in removal of phenols (Figure 5), biomass washout rate was measured. Biomass concentration in the reactor was greater than hydraulic wash-out rate, thus phenols removal was occurring and was not only a result of delution in the reactors but results of both biosorption and oxidation. The oxidation has been proved from analyses of laccase enzyme concentration (Figure 6) that was following a similar trend as phenols removal rate. According to biomass concentration measurements in non-sterile conditions experiments in a batch mode, specific growth rate was calculated using linearized Monod Equation (1). We found that maximum specific
growth rate ($\mu_{\text{max}}$) of fungi biomass for TP removal was 0.075 (1/h) and half-saturation coefficient $K_s = 0.10$ (g/L). Maximum specific growth rate ($\mu$) – 0.075, was slower than conventional bacterial rate, but considering toxicity and removal complicity of TP, still is acceptable for removal of TP at concentration of 100 mg/L. The shift of one day can be explained by inertia between enzyme production and TP final concentration reduction. In Figure 7 are F/M graphics, where food (F) is the concentration of TP and mass (M) is the concentration of fungal biomass in the reactor. This ration shows that about 8–10 mg of phenols are removed by milligrams of fungi, which indicates a need to maintain relatively high biomass concentration in the reactor to accommodate peak loads of phenols.

Phenolic compounds can be treated with conventional biological methods, which are friendly and energy saving, but it cannot treat high concentration, above 0–20 mg/L of TP. Meanwhile chemical methods can deal with medium and high concentrations (20–200 mg/L), but involves use of chemicals, which subject environment pollution in long term and of global chemical use (Villegas et al. 2016). Current studies confirmed that fungi bioreactors can be practically used, in real (non-sterile) conditions, also in as pre-treatment stage for specific TP removal, before existing wastewater treatment plant (WWTP) reactors.

In further, studies should be focused on TP removal process stabilization with constant removal rate. This could be done by bio-augmentation process, by dosing fixed amount of fungal biomass in reactor. According to current results, increase of biomass concentration gives proportional increase also for degradation rate of TP, as seen in Figure 8. Stabilization of TP removal rate could be done by constant
supplementation of fungal biomass with peristaltic pump or by manual adding of biomass volume in fixed time steps.

Comparing to other conventional TP removal methods (distillation, absorption, extraction, chemical oxidation, and electrochemical oxidation) and advanced removal methods (Fenton processes, ozonation, wet air oxidation, and photochemical treatment) fungal bioreactor approach use less chemicals, in same time, has at low energy consumption, since it is biological treatment method.

Therefore, TP removal technology with *T. versicolor* bioreactors looks promising for practical appliance in industrial wastewater treatment, in both engineering and economical point of view. TP removal is done by laccase enzyme activity, which is one of the major fungal enzymes for cyclic compound cleavage. Biodegradation mechanism is based on the stroke of the enzymes such as laccase, lignin peroxidase, NADH-DCIP reductase, tyrosinase, hexane oxidase and amipropyrine N-demethylase (Solis et al. 2012). An earlier study of Wong & Yu (1999) proposed a mechanism for the increased detoxification capacity of *T. versicolor* laccase, that involves the decolourization of non-substrate phenol dyes in effluents via substrate which also act as mediators in the laccase catalytic cycle (Wesenberg et al. 2005).

CONCLUSIONS

The following conclusions were drawn from the study:

- High concentration of phenols in wastewater can be effectively removed both in batch and continuous reactors using fungus-augmentation approach.
- From current studies, maximum specific growth rate (µ) was calculated to be 0.075 (1/h), for TP removal in non-sterile conditions, at continuous flow mode.
- Fungal biomass reactors can be used as a pre-treatment stage for TP and toxicity removal, before existing WWTP bacteria bioreactors.

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REFERENCES


