Simultaneous biodegradation of methylene blue and phenol by *Trametes hirsuta* in batch and packed bed reactors

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Abstract
Fungal biodegradation is widely described as an efficient technological process for wastewater treatment, especially for removal of recalcitrant pollutants. Treatment of wastewater containing methylene blue and phenol has received much attention in recent years due to their toxicities and slow biodegradation. In this work, Trametes hirsuta mycelium was grown and used to remove methylene blue and phenol. Single and binary removal of both pollutants was carried out in batch and fixed bed column. Removal mechanism was investigated using active and inactive T. hirsuta and crude growth medium.

Results showed that at methylene blue and phenol concentrations of 25 and 10 mg/L respectively, removal of more than 80% of both products in binary batch system was achieved within only 80 minutes. Passive adsorption mechanism was slightly involved in methylene blue removal in single system while the removal of phenol was only possible through both intracellular and extracellular enzymatic degradation.

Keywords: Methylene blue, phenol, biodegradation, *Trametes hirsuta*, batch, packed bed reactor.

Introduction
The problem of aquatic pollution is closely linked with the industrial development. Industrial and municipal wastewater contains many chemicals that negatively affect living organisms. Organic micropollutants in wastewater cause oxygen depletion and have severe consequences for the stream biota[1]. Dyes and phenolic compounds are well designated as being among the most known toxic environmental priority pollutants[2].

Among others, phenol and methylene blue are organic micropollutants with high presence frequency. Phenol enters the environment as intermediates during the biodegradation of natural polymers containing aromatic rings such as lignin and tannin[3]. Even at low concentrations, phenol is considered as a priority pollutant since it is harmful to organisms[4]. Inhalation and dermal exposure to phenol is highly irritating to the skin, eyes and mucous membranes[5]. Phenol transformation under natural conditions can lead to the formation of many toxic substituted phenols. Substituted phenols toxicity is mainly of two types: the toxicity due to phenoxyl radical formation and the toxicity caused by metabolites[6].

Environmental impact of dyes has also attracted attention recently. Approximately 0.7 million tons of synthetic dyes are generated annually. However, at least 15% of these dyes is lost during the dyeing process and released into the environment via industrial wastewater-discharge processes[7]. Methylene blue is one of the most commonly used dyes for natural fibers such as cotton or silk. Direct contact can cause eye burns, nausea, vomiting and mental confusion[8]. These toxic and even carcinogenic pollutants are posing serious hazard to aquatic living organisms. Thus, it is necessary to eliminate them from wastewater before its discharge.

Innovations in environmental sciences and engineering play a significant role in environmental protection. Conversion of contaminated wastewater to harmless and readily disposable water is of crucial importance because of the fast expansion of fresh water consumption worldwide[9]. Several technologies have been proposed for the removal of phenol and/or methylene blue from wastewater. These include membrane filtration[10], electrocoagulation/flotation[11] and advanced oxidation processes.

Although chemical processes can be easily and effectively used as treatment methods, the use of chemicals in solution would inevitably introduce secondary contamination due to their toxic nature to human beings and the eco-system[13]. Moreover, the pollution complexity in wastewaters makes treatment more difficult because of the specificity of such treatments. Biological treatment of micropollutants was found to be an attractive alternative to replace or supplement conventional technologies. These include biosorption and/or biotransformation. The major advantages of these bioprocesses are good removal performance, low costs, flexibility, simplicity of design and biocompatibility[14]. Many kinds of biological materials like algae[15], fungi[16] and bacteria[17,18] were used for the degradation of organic micropollutants. Unlike bacteria in conventional wastewater treatment systems, white-rot fungi (WRF) can degrade wide spectrum of resistant compounds including textile dyes by

non-specific extracellular enzymes[19]. Some WRF have shown the ability to remove and mineralize dyes and phenolic compounds in a more competent way than bacteria[20,21]. The transformation products generated by fungal transformation are typically less toxic, or show lower biological activity than the parent compounds[22].

Fungal treatment of wastewaters was described as a promising technology because of the unspecific ligninolytic enzymes[23], this includes laccase (EC 1.10.3.2), manganese peroxidase (EC 1.11.1.13) and lignin peroxidase (EC1.11.1.14)[24]. Laccase is a copper-containing enzyme requiring only O_2 for its catalytic transformation of numerous substrates[25]. The enzyme can effectively catalyze the oxidation of a large spectrum of phenolic compounds[26]. Laccase oxidative capacity can be expanded to the non-phenolic compounds by introducing some mediators. Even in multicompounds solutions, Laccase Mediated Systems (LMS) resulted in a high oxidation capability[27]. LMS have attracted a particular interest for their versatility [28]. Recently, the WRF *Trametes hirsuta* was described as a promising laccase producer[29-31]. It needs only a simple, defined medium for rapid growth in suspension culture and it could be a good alternative to remove recalcitrant micropollutants from wastewaters.

The aim of this work was to use *T. hirsuta* to remove phenol and methylene blue from spiked mixed solutions. The effect of operational conditions (contact time, initial concentration, single and binary mixtures) on removal efficiency was studied. Zero, first and second order kinetic models were tested to fit experimental data. Phenol and methylene blue removal mechanism was then discussed according to the experimental results.

Material and Methods

Fungal culture: *Trametes hirsuta* was purchased from the Cultures Collection of the Durmishidze Institute of Biochemistry and Biotechnology (Tbilisi, Georgia). It was maintained on potato dextrose agar (PDA) plates at -4°C and subcultured every 3 months. 90 mg of *T. hirsuta* mycelium were mixed with 100 ml of Malt-Glucose-Yeast extract (MGY) growth medium. The latter was prepared by dissolving 10% (w/v) malt extract, 4% (w/v) glucose and 4 % (w/v) yeast extract in Milli-Q water. The mixture was then orbitally agitated at 150 rpm during 16 days for mycelium proliferation. Laccase and manganese peroxidase activities were monitored during the whole culture period of 16 days. The pH of the growth medium was maintained at 7 for all conditions. This pH was selected based on laccase maximal activity and fungal growth performance described previously[29].

Methylene blue and phenol quantifications: Methylene blue and phenol solutions were filtered with Whatmann filter paper (VWR, France). Methylene blue quantification was carried out directly with a UV-Visible spectrophotometer (ULTRASPEC 1000, Amersham Pharmacia, USA) at 668 nm. Phenol concentration was determined by 4-aminoantipyrine method at 500 nm[32]. UV quartz cuvettes were used for quantification. Standard curves were established daily and used to extrapolate unknown concentrations.

Single removal of methylene blue and phenol by active *Trametes hirsute*: Single removal experiments were conducted in batch mode. 100 ml of MGY medium containing developed mycelium of *T. hirsuta* were mixed in 250 mL Erlenmeyers with solutions of methylene blue or phenol under sterile conditions. Initial concentrations were varied from 25 to 100 mg/L for methylene blue and from 10 to 50 mg/L for phenol. These concentrations are relevant to the real environmental conditions[33,34]. Initial pH was adjusted to 7 with 0.1N HCl and/or 0.1N NaOH, temperature was maintained at 25°C. The flasks were agitated for 6 h at 150 rpm. The residual methylene blue and phenol concentrations were determined as function of time.

The removal of both pollutants R (%) was calculated using equation 1:

$$R(\%) = \frac{(C_i - C_t)}{C_i} 100 \quad (1)$$

where C_i is the initial concentrations of methylene blue or phenol and C_t corresponds to the residual concentrations of methylene blue or phenol at time t.

All experiments were performed in triplicate and the reported data were presented as mean ± standard errors; *T. hirsuta* free solutions were kept under stirring and used as blanks.

Binary removal of methylene blue and phenol by active *Trametes hirsute*: Binary removal experiments of methylene blue were carried out by mixing 100 ml of MGY medium containing developed mycelium of *T. hirsuta* with 10mg/L of phenol and various concentrations of methylene blue (from 25 to 100 mg/L).

In the same way, phenol removal in binary system was conducted by mixing 10 mg/L of methylene blue with various concentrations of phenol (from 10 to 50 mg/L).

After 6 hours of contact, samples were filtrated for final concentrations determination, then removals of both methylene blue and phenol were calculated using equation 1.

Single and binary removal of methylene blue and phenol by inactive *Trametes hirsute*: The inactivated form of *T. hirsuta* was used to determine the biosorption contribution to both pollutants removal in single and binary mixtures. *T. hirsuta* inactivation was carried out by autoclaving developed fungal mycelium at 120°C for 15 minutes. Initial concentrations of 25mg/L of methylene blue and/or 10 mg/L of phenol were mixed with inactive fungus. The mixtures

were agitated at 150 rpm; then, samples were withdrawn and filtered at regularly periods of time for analysis.

Single and binary removal of methylene blue and phenol by crude growth medium without *T. hirsuta* cells: To understand the mechanisms involved in pollutants removal by *T. hirsuta*, the study of crude enzyme extracts contribution (extracellular content without fungus cells) is of crucial importance[35,36].

Removal of both methylene blue and phenol from single and binary mixtures was achieved in batch mode. 16 days old cultures of *T. hirsuta* were filtered at 0.5 µm using filter paper under sterile conditions. 100 mL of the crude filtrate were placed in 250 mL Erlenmeyer flasks and mixed with both pollutants to reach final concentrations of 25 mg/L for methylene blue and 10 mg/L for phenol. Every 10 minutes, 2 mL samples were withdrawn, filtered and analyzed to calculate methylene blue and phenol removals.

Kinetic approach: Zero, first and second models were applied to the residual concentrations of methylene blue or phenol in batch incubation period in order to detect the substrate degradation kinetic model. To do that, the curves of (*C*) versus time (*t*), ln *C* versus *t* and *1/C* versus *t* were plotted in order to determine the best fitting kinetic model. The graphical application of kinetic models was carried out according to equations (2-4)[37].

$$C_t = C_i - k_0 t \quad (2)$$

$$\ln\left(\frac{C_i}{C_f}\right) = k_1 t \quad (3)$$

$$\frac{1}{C_t} - \frac{1}{C_i} = k_2 t \quad (4)$$

where k_0, k_1 and k_2 are the zero, first and second kinetics rate constants respectively.

Removal of methylene blue and phenol with *T. hirsuta* in a packed bed reactor: To test the ability of *T. hirsuta* to remove both pollutants in continuous mode, a packed bed reactor (PBR) filled with active *T. hirsuta* was used. The PBR consisted of a glass column with an internal diameter of 1.5 cm and a height of 25 cm. The PBR was filled with 10 g of *T. hirsuta*. 25 mg/L solutions of methylene blue and/or 100 mg/L solution of phenol were pumped at a flow rate of 1mL/min in an up-flow mode.

The column was maintained in a temperature-controlled room at 25°C. Samples of the outlet solutions were withdrawn every 10 minutes; methylene blue and phenol quantification were processed.

To investigate the adsorption contribution in both methylene blue and phenol removals, PBR experiments were carried out at the same operation conditions with inactive *T. hirsuta*.

Results and Discussion

As described in a previous work[29], *T. hirsuta* secreted only laccase. No Mn Peroxidase activity was detected in all cultures under our experimental culture conditions.

Single removal of methylene blue with active *T. hirsuta*: Removal of methylene blue by active *T. hirsuta* in single batch mode is shown in fig. 1. It reached 70% for the initial concentrations 25 mg/L and 50 mg/L and less than 10% for the initial concentration of 100 mg/L. This indicates that methylene blue removal decreases with increasing initial concentration, this can be probably explained by a toxic effect of methylene blue on active fungus[38]. The transformation seems to be rapid within the first 2 hours of contact, thereafter; it becomes markedly steady. This could be due to the accumulation of oxidation products generated in the mixture leading to a partial inhibition of laccase activity[39].

Some recent studies reported that laccase from *T. hirsuta* is able to remove a wide range of chemicals such as pharmaceuticals[35], synthetic dyes[40] and dihydroquercetin [41]. Multiple mechanisms may be involved in this removal processes such as adsorption, extracellular transformation and intracellular absorption.

Single removal of phenol with active *T. hirsute*: At phenol initial concentration of 30 mg/L, 80% of phenol removal was reached within one and half hours (Fig. 2) indicating that *T. hirsuta* is more efficient to remove phenol than methylene blue. High concentrations of phenol did not have significant effect on its removal. At 50mg/L of phenol, about 75% was removed within two hours of treatment. Consequently, our results are quite interesting since (a) phenol is typically more recalcitrant to degradation than methylene blue[42] and (b) phenol is more toxic in aqueous environments than methylene blue according to the US Environmental Protection Agency (EPA).

Phenol removal by active *T. hirsuta* can be explained by laccase oxidation. Laccase is a non-substrate specific copper-containing phenoloxidases requiring only dioxygen as cofactor for the catalytic oxidation of phenolic compounds. Its oxidation reaction leads to release of water as by-product and free reactive phenoxy radicals, these radicals have the trend to form polymeric structures less reactive and are less toxic than the phenol[25].

Binary removal of methylene blue with active *T. hirsute*: Active *T. hirsuta* was efficient to remove methylene blue in presence of 10 mg/L of phenol (Fig. 3). In comparison with single system, binary removal was more rapid, removal of 80% was reached within only one hour, while 3 hours were needed to remove the same percentage in single system (Fig. 1). On the other hand, at high concentration of 100 mg/L, 50% of methylene blue was removed within 60 minutes (Fig. 3), while only less than 10% was removed in single system. This can probably be explained by a mediated removal of

methylene blue by laccase enzyme in presence of phenol. Laccase Mediated Systems (LMS) were extensively studied to improve laccase transformation abilities[43]. Since laccase was restricted to phenolic compounds due to its low oxidation potentials[44], LMS resulted in a high oxidation range, increasing the number of degradable products to non-phenolic compounds such as methylene blue[45].

Binary removal of phenol with active *T. hirsute*: Phenol removal in binary system was more rapid than in single system (Fig. 4). 80% of removal was reached at about 60 minutes of contact which indicates a synergistic interaction between phenol transformation products and methylene blue. Haroune et al[27] obtained a removal of about 40% of carbamazepine in a cocktail solution of 17 pharmaceuticals using *T. hirsuta*. They suggested that some phenolic compounds present in solution like acetaminophen, can act as electron shuttles. In our study, we suggest that phenol is the electron shuttle during methylene blue removal.

Kinetics of methylene blue and phenol removal: The kinetic rate constants and correlation coefficients relevant to the zero, first and second orders are summarized in table 1. In both single and binary systems, the comparison of regression coefficients (R^2) R^2>0.95 relevant to zero-, first- and second-order rate constants (k_0, k_1 and k_2 values) showed that methylene blue and phenol degradation could be described by both first and second order models while the first order model fits better the experimental data. Potthast et al [46] used zero, first and second-order kinetic models to describe benzyl alcohols oxidation with laccase mediated system. They found that the first phase of oxidation followed zero-order kinetics while in the second phase, oxygen consumption was considerably slower and followed second-order kinetics.

Similarly, Olejnik et al tested the photodegradation of methylene blue and phenol by a mesoporous silica supports incorporated with titanium (Ti/SBA-15). They found that the first order kinetics fitted well the experimental data, the rates constants were found to be 7.9 10^{-2} min^{-1} and 1.6 10^{-2} min^{-1} for both pollutants respectively. Consequently, the methylene blue and phenol were totally removed after 40 and 180 min respectively.

According to the first order rate constants illustrated in table 1, the biodegradation of methylene blue and phenol with *T. hirsuta* is more rapid in binary mixtures than in single solutions, this indicates the positive effect of mediated removal of both pollutants.

Single and binary removal of methylene blue and phenol with inactive *T. hirsute*: It is well known that fungal surface biosorption capacity is a key factor for the degradation rate in biodegradation system[38]. Results indicated that the adsorption contribution during methylene blue removal from single solution was less than 20% (Fig. 5), this removal corresponds to an adsorption capacity of 5 mg/g. This is a very low value compared to other adsorbents[8,47]. Although methylene blue was slightly removed in single system, both methylene blue and phenol have not been removed in binary system, this could be due to a low affinity toward *T. hirsuta* surface, resulting in a small contribution of passive adsorption during methylene blue and phenol removal by *T. hirsuta* fungi. Similarly, Batista-García et al[48] found that the adsorption contribution was less than 7% during the removal of phenol with a number of fungal strains belonging to the *ascomycota*, *basidiomycota* and *zygomycota* genera.

Single and binary removal of methylene blue and phenol by crude growth medium without *T. hirsuta* cells: Methylene blue and phenol removal was obviously remarkable with only growth medium; maximum removal was reached after about 100 minutes of contact, suggesting that removal with growth medium alone was slightly slower than with the whole alive cells (Fig. 6). For single mixtures, about 70% of micropollutants were removed, while in binary system, removal of about 80% was observed in the same period of time. These results indicate the significant extracellular enzymatic contribution in pollutants removal; however, it seems that it is not the only involved mechanism. The removal with active fungi was slightly more rapid and more efficient indicating that elimination of both methylene blue and phenol was not exclusively conducted with extracellular oxidation.

Similar results were found by Rodríguez et al[31] during the removal of Indigo Carmine and Lanaset Marine dyes by *T. hirsuta*. They observed that higher decolorization percentage was achieved with actively growing cultures of *T. hirsuta* than with the culture filtrate. Moreover, results obtained after inhibition of *T. hirsuta* growth by antibiotics indicated that dye decolorization could not exclusively be attributed to laccase activity but also to other growth associated mechanisms related with intracellular enzymes. The same phenomena was observed by Haroune et al[35] during pharmaceutical removal by *T. hirsuta*.

Single and binary removal of methylene blue and phenol in a packed bed reactor with active and inactive *T. hirsute*: *T. hirsuta* was more efficient in binary system (Fig. 7). In single system the active fungus removed about 40% of methylene blue and only 50% of phenol, while in binary system almost the totality of methylene blue and phenol was removed by active fungus within first two hours of treatment. This confirms the results obtained in batch mode and can be explained by a synergistic interaction between the two pollutants. Phenol, as a good substrate for laccase enzyme, plays probably a mediator role during removal processes which increases both phenol and methylene blue removal in packed bed column. Removal of pollutants from binary mixture in packed bed reactor with inactive cells showed that less than 35% of methylene blue and about 15 % of phenol were removed indicating that adsorption has no significant contribution during treatment process.

These results are in consistency with those found in batch mode, suggesting that removal of methylene blue and phenol was realized by an enzymatic process. The same phenomena was deeply investigated by Haroune et al.[35] They suggested that during the removal of four nonsteroidal anti-inflammatories products (ibuprofen, indomethacin, ketoprofen and naproxen) by *T. hirsuta*, a removal of about 100% was achieved in the presence of whole cells, while less than 40% removal was reached using crude extract. This suggests that other mechanisms such as active uptake and intracellular enzymes could contribute to the observed removal. Extracellular enzymatic degradation was also applicable during removal of other pharmaceuticals such as acetaminophen and mefenamic acid.

Table 1
Kinetic parameters of methylene blue and phenol removal by active *T. hirsute*

Removal system	Initial concentration (mg/L)	Zero order model		First order model		Second order model	
		k_0 (mg/L.min)	R^2	k_1 (1/min)	R^2	k_2 (L/mg.min)	R^2
Methylene Blue single	25	8.8×10^{-2}	0.879	7.6×10^{-3}	0.973	1.3×10^{-2}	0.994
	50	1.8×10^{-1}	0.974	5.9×10^{-3}	0.970	9.0×10^{-3}	0.943
	100	3.4×10^{-2}	0.324	3.9×10^{-4}	0.952	4.1×10^{-4}	0.339
Phenol single	10	6.4×10^{-2}	0.681	4.3×10^{-2}	0.998	1.0×10^{-1}	0.935
	20	1.3×10^{-1}	0.654	3.1×10^{-2}	0.939	6.5×10^{-2}	0.913
	30	1.7×10^{-1}	0.703	2.4×10^{-2}	0.945	5.3×10^{-2}	0.977
	40	2.6×10^{-1}	0.916	1.6×10^{-2}	0.994	3.1×10^{-2}	0.934
	50	3.2×10^{-1}	0.937	1.3×10^{-2}	0.991	2.4×10^{-2}	0.945
Methylene Blue binary	10	1.2×10^{-1}	0.940	2.5×10^{-2}	0.973	4.3×10^{-2}	0.928
	20	2.2×10^{-1}	0.910	2.6×10^{-2}	0.986	4.9×10^{-2}	0.967
	30	3.1×10^{-1}	0.978	1.8×10^{-2}	0.983	2.9×10^{-2}	0.945
	40	4.4×10^{-1}	0.887	2.8×10^{-2}	0.985	5.3×10^{-2}	0.977
	50	6.3×10^{-1}	0.961	2.7×10^{-2}	0.987	4.7×10^{-2}	0.919
	100	6.8×10^{-1}	0.803	1.2×10^{-2}	0.884	1.9×10^{-2}	0.945
Phenol binary	10	1.2×10^{-1}	0.938	2.4×10^{-2}	0.973	4.3×10^{-2}	0.929
	20	2.3×10^{-1}	0.907	2.7×10^{-2}	0.992	4.9×10^{-2}	0.968
	30	3.5×10^{-1}	0.962	2.5×10^{-2}	0.992	4.4×10^{-2}	0.947
	40	4.8×10^{-1}	0.836	3.4×10^{-2}	0.977	6.3×10^{-2}	0.935
	50	6.4×10^{-1}	0.913	3.2×10^{-2}	0.992	5.9×10^{-2}	0.929

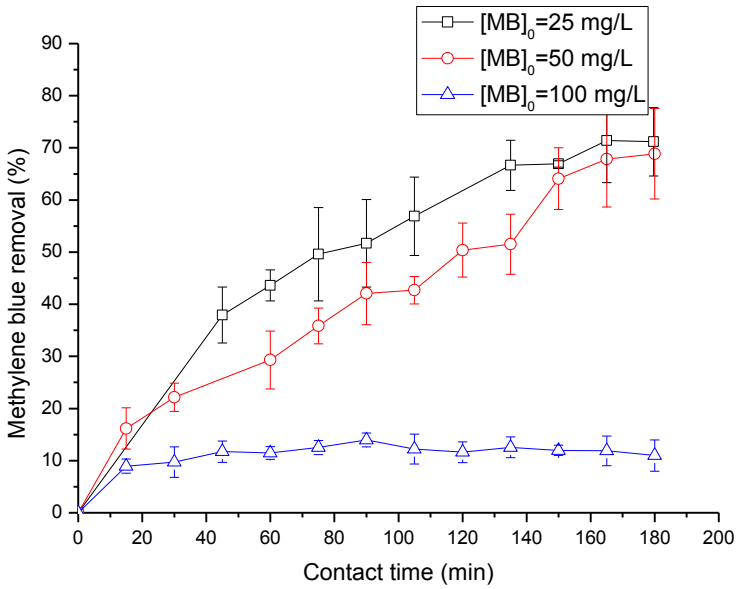

Fig. 1: Single removal of methylene blue (MB) in batch mode with active *T. hirsuta* (*T. hirsuta* quantity, 100 mg of dry mass; pH, 7; Agitation speed, 150 rpm; Temperature, 25°C)

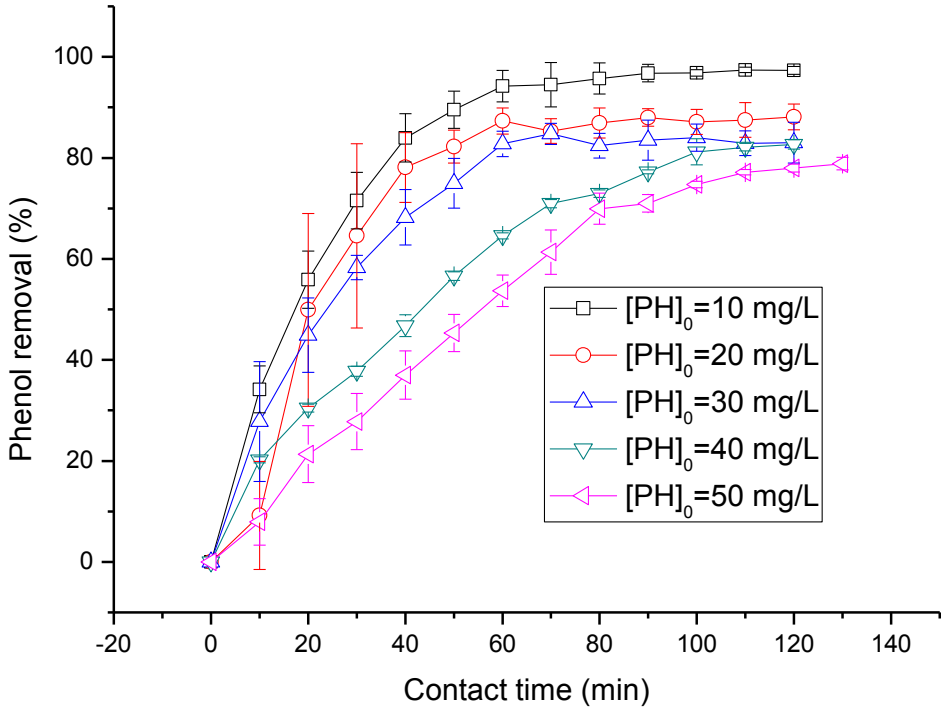

Fig. 2: Single removal of phenol (PH) in batch mode with active *T. hirsuta* (*T. hirsuta* quantity, 100 mg of dry mass; pH, 7; Agitation speed, 150 rpm; Temperature, 25°C)

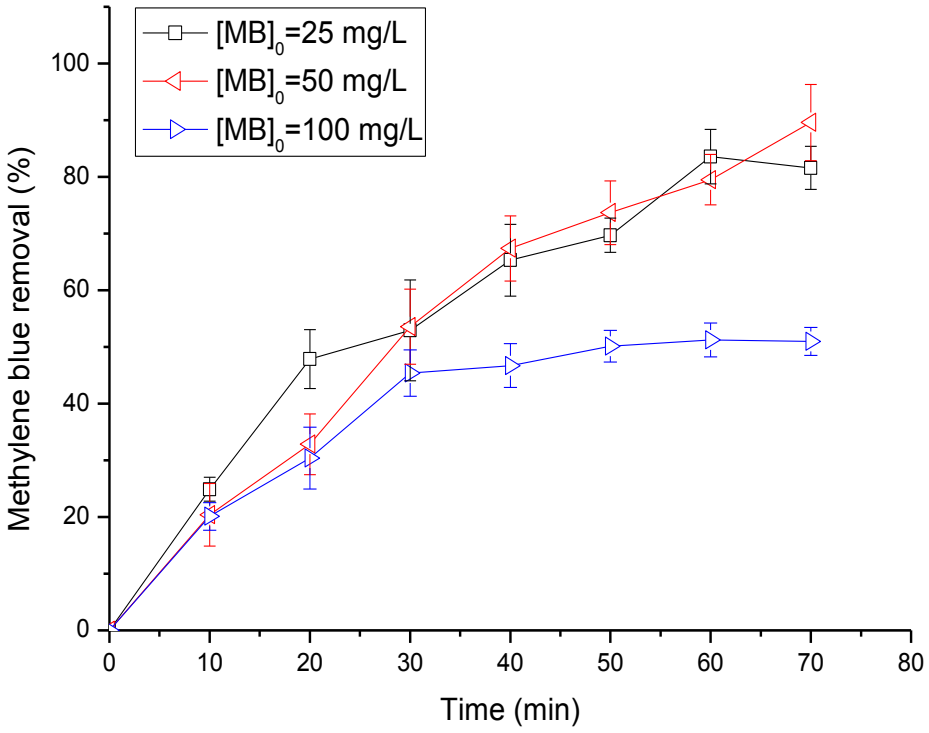

Fig. 3: Binary removal of methylene blue (MB) in batch mode with active *T. hirsuta* ($[PH]_0$,10 mg/L; *T. hirsuta* quantity, 100 mg of dry mass; pH, 7; Agitation speed, 150 rpm; Temperature, 25°C)

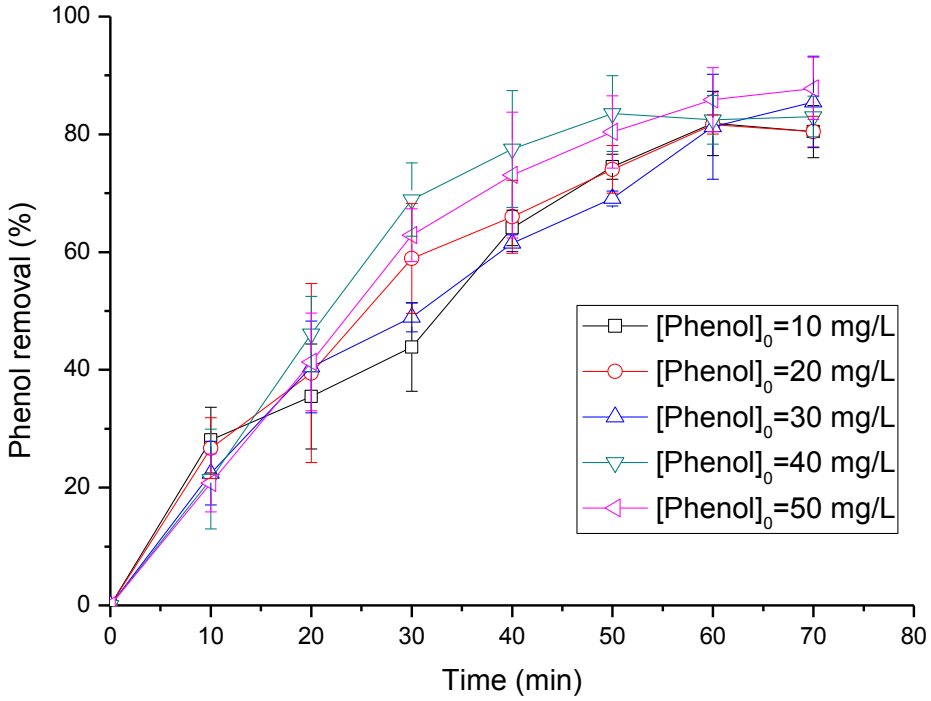

Fig. 4: Binary removal of phenol (PH) in batch mode with active *T. hirsuta* ([MB]$_0$,10 mg/L; *T. hirsuta* quantity, 100 mg of dry mass; pH, 7; Agitation speed, 150 rpm; Temperature, 25°C)

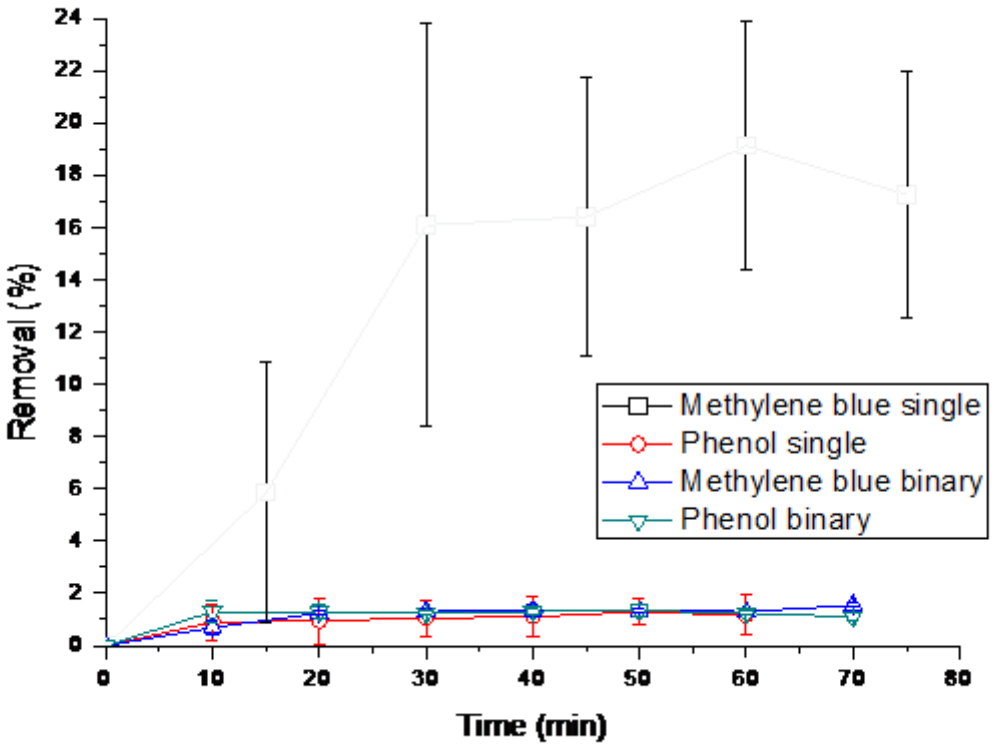

Fig. 5: Single and binary removal of methylene blue (MB) and phenol (PH) in batch mode with inactive *T. hirsuta* ([MB]$_0$, 25 mg/L; [PH]$_0$,10 mg/L; *T. hirsuta* quantity, 100 mg of dry mass; pH, 7; Agitation speed, 150 rpm; Temperature, 25°C)

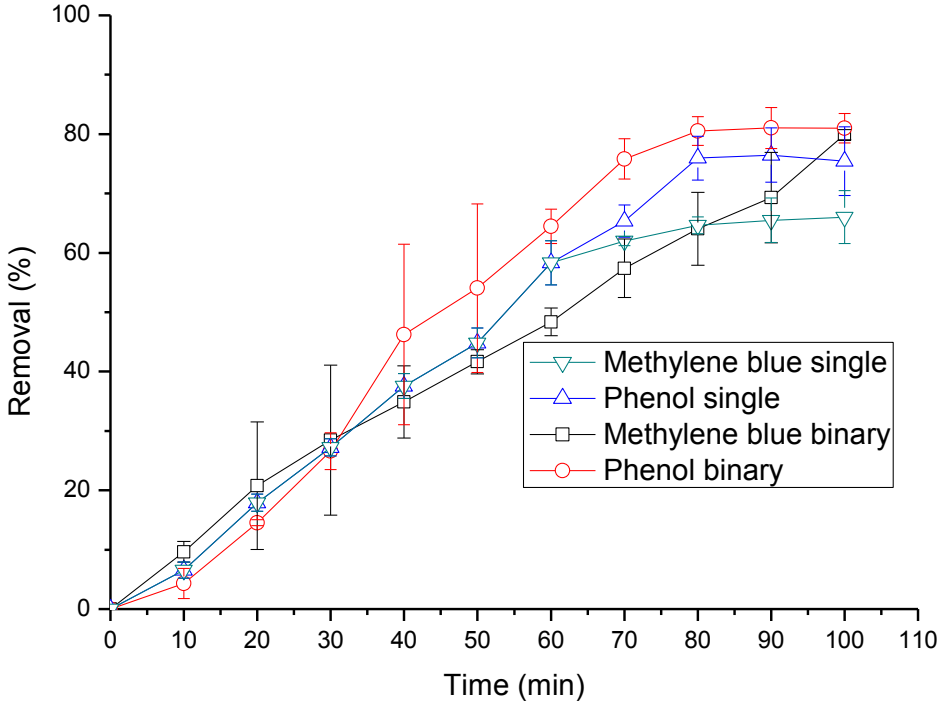

Fig. 6: Single and binary removal of methylene blue (MB) and phenol (PH) in batch mode with crude growth medium without fungi ([MB]$_0$,25 mg/L; [PH]$_0$,10 mg/L; pH, 7; Agitation speed, 150 rpm; Temperature, 25°C)

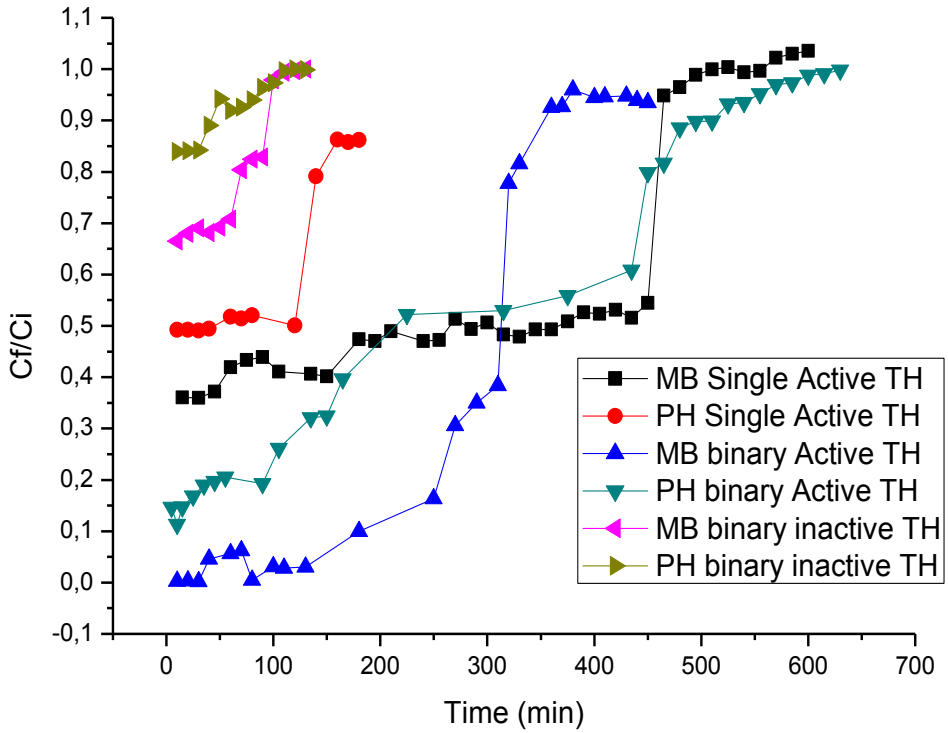

Fig. 7: Single and binary removal of methylene blue (MB) and phenol (PH) in backed bed column mode with active and inactive *T. hirsuta* ([MB]$_0$,25 mg/L; [PH]$_0$,10 mg/L; *T. hirsuta* quantity, 1 g of dry mass; pH, 7; flow rate, 1 mL/min; bed high, 15 cm; Temperature, 25°C)

Conclusion

Active *Trametes hirsuta* was able to remove methylene blue and phenol rapidly in batch and column modes. Experiments showed that the fungus was more efficient in binary mixtures, where more than 80% of both molecules disappeared within one hour of treatment in batch mode. Adsorption onto inactive *T. hirsuta* was not significant, less than 20% of both pollutants were removed by adsorption, while removal by crude growth medium reached 80% in less than 70 minutes. This indicates that the removal was achieved with an enzymatic transformation which includes extracellular and/or intracellular enzymes.

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