

Factors Governing Degradation of Phenol in Pharmaceutical Wastewater by White-rot Fungi: A Batch Study

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Abstract: Phenol is a major contaminant in the industrial water effluent, including pharmaceutical wastewaters. Although several physico-chemical methods for removal of phenol exist, they are of high cost, low efficiency, and generate toxic by-products. Thus, there is a need to develop technologies for biological removal of phenol from wastewater. In this study, the degradation of phenol in pharmaceutical wastewater by monoculture of white-rot fungi was studied. The degradation rate of total phenol in batch flasks by four fungal monocultures of *Trametes versicolor*, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* and *Irpex lacteus* in synthetic medium was compared. The results showed that white-rot fungus *T. Versicolor* was the most effective of the species. Further selection tests of optimal conditions of biomass concentration, pH and temperature were done, indicating that optimal conditions of degradation are at pH 5-6, temperature 25 °C, and biomass inoculum 10% (v/v). Under optimal conditions, total phenol was reduced by 93%, concentration of total phenol decreasing from 420±12 mg/l to 29±1 mg/l in seven days, with *T. Versicolor* specie. This study suggested that biological treatment with fungi may effectively be used as a pre-treatment stage for removal of phenol before polishing wastewater with conventional biological methods.

Keywords: Biodegradation, Phenol removal, *Trametes versicolor*.

1. INTRODUCTION

Phenol is present in effluents from major chemical and pharmaceutical industries such as petrochemical industries, petroleum refineries, coal gasification operations, liquefaction process, resin manufacturing industries, dye synthesis units, pulp and paper mills and pharmaceutical industries [1], total phenol concentration being in range 300 – 400 mg/l [2, 3]. It is a highly corrosive and nerve poisoning agent. Ingestion of phenol contaminated products causes harmful side effects such as dyspnoea, gastrointestinal effects, and liver and kidney damage in long-term exposure [4]. In Europe alone more than 1.6 million tons of phenol is consumed in manufacturing annually from which 11 200 tons are discharged in environment due not sufficient wastewater treatment [5]. Phenolic compounds are cyclic molecular structure, therefore their degradation in the environment is slow, causing their accumulation in effluent environment.

Various physicochemical processes, like activated carbon adsorption, solvent extraction, chemical oxidation and electrochemical methods have been developed to remove phenolic compounds from wastewaters [6-12]. However, wide applications of some of these remediation strategies have been limited due to problems such as high cost, low

efficiency, and generation of toxic by-products [13, 14]. Biological treatment with activated sludge, membrane reactors or trickling filters, have been tested with variable degree of success [15]. The main limitation of these conventional biological methods is use of bacteria as dominating biological agents in these systems, although they are not naturally designed for so strong bound break down and inhibited at high concentrations of phenol.

Fungi successfully can be used for phenol degradation since they are less sensitive to inhibition [16] and, due to active production of various enzymes [17], which are able to degrade cyclic ring compounds. One of most effective cyclic compound cleaving fungi classes is basidiomycetes, because of featuring lignin biodegradation enzymes: lignin peroxidases, manganese peroxidases, laccase. In phenol biodegradation these are the key enzymes, which oxidase phenol by two consecutive one-electron oxidation steps with intermediate cation radical formation [18].

There are reports about ability to degrade cyclic compounds from cooking industry wastewater by 87% (from 313 mg/l) in six days, from olive mill waste waters by 89% (from 400 mg/l) in five days [2, 3]. However, there is no comprehensive study about optimal conditions of phenol elimination from pharmaceutical wastewater and their quantitative effect degradation rate. Moreover, to develop a treatment technology using fungi species, environmental condition impact on degradation process should be tested also outside the optimum range.

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The aim of this study is to determine phenol degradation rate and optimal conditions for four basidiomycetes which are *T.Versicolor*, *P.Chrysosporium*, *G.Trabeum* and *I.Lacteus*, having enzymes able to degrade cyclic compounds. The most effective fungi species, as well variation tests of inoculum biomass, pH and temperature were studied in batch scale with monocultures using synthetic pharmaceutical wastewater.

2. MATERIAL AND METHODS

2.1. Pharmaceutical Wastewater Collect Ion and Characterization

The solution with phenols was obtained from a toxic tank of pharmaceutical waste water treatment plant (WWTP) site. The solution was added to biological bioreactors by dosing small amount (dilution up to 1:1000) and mixing it with non-phenol containing waste water from other manufacturing sites.

2.2. Pharmaceutical Wastewater Supplementation

The synthetic wastewater was prepared of the solution from pharmaceutical waste water treatment plant (WWTP) toxic buffer tank by adding necessary macro elements, KH_2PO_4 - 0.80 g/l, K_2HPO_4 - 0.20 g/l, MgSO_4 - 0.50 g/l, Yeast extract - 5.00 g/l, NH_4NO_3 - 3.00 g/l (medium A). The medium was autoclaved at 121°C for 15 min, before autoclave pH was corrected to pH 5.5, using 1M HCl. After autoclaving the phenol wastewater from WWTP was added to solution, with final concentration of total phenol 600 mg/l and COD 9 000 mg/l.

2.3. Preparation of Fungi Stock Cultures

In screening experiments the following basidiomycete fungi's species were used: *T.Versicolor*, *P.Chrysosporium*, *I.Lacteus* representing white-rot fungi's and *G.Trabeum* as brown-rot fungi. Stock cultures were kept on sterile agar plates at refrigerator temperature of 5 °C.

Before phenol degradation batch experiments stock culture from agar plate were inoculated in liquid medium. 150 ml of medium B (KH_2PO_4 - 0.80 g/l, K_2HPO_4 - 0.20 g/l, MgSO_4 - 0.50 g/l, Yeast extract - 5.00 g/l, NH_4NO_3 - 3.00 g/l, Dextrose - 3.00 g/l, pH = 5.5;) was filled in 250 ml flasks and pH was corrected to pH 5.0, using 1M HCl. Then flasks were covered with cotton corks, and autoclaved at 121 °C for 15 min. After autoclave flasks were placed cooled down to room temperature. Stock culture inoculation to flask medium was done in fume hood, prior making disinfection of inner surface by UV light for 30 min. Flasks of inoculated medium were placed in thermostat with orbital shaker at 30 °C and 150 rpm for 7 days. Afterwards flask medium was homogenized with prior autoclaved glass spheres 2 mm in diameter.

2.4. Batch Test Description

Flasks of 250 ml volume covered by cotton cork were used for batch tests. Synthetic medium A was filled up to 125 ml, then autoclaved and cooled down to room temperature. After that 8 ml of phenol concentrate obtained

from WWTP was added to medium and shacked, afterwards 15 ml of homogenized stock culture was added to experiment volume (OD=0.300 Abs at 600 nm). Stock culture was previously cultivated in liquid medium B. Prepared flasks closed with cotton corks were placed in thermostat with orbital shaker at 30 °C and 150 rpm. After screening test of four fungi species (with conditions $t=30^\circ\text{C}$, pH=5.0, v/v biomass = 30%, total phenol = 600 mg/l), most effective fungal specie were selected for further optimal conditions experiments. In conditions experiment parameters were varied in these steps: for temperature 20, 25, 30, 35 °C, for pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, for stock culture aqueous (OD=0.300 Abs at 600 nm) biomass v/v 5, 10, 20, 30%. After screening tests and optimal biomass concentration experiments total phenol concentration in experiment medium were decreased from 605 to 420 mg/l, maintaining 30% safe reserve of maximal phenol load for temperature and pH variations experiments. All batch tests were done in three repetitions. Statistics were calculated with MS Excel, using average and standard deviation (SD) functions.

2.5. Sampling and Analyses

All samples from flasks were taken in fume hood, prior inner surface of fume hood were disinfected with UV light for 30 min. Samples were taken with sterile syringe and filtered through 0.45 µm filter (Minisart, high flow syringe filters), to determine soluble COD and phenol concentration, and ensure concentration retention of current experiment moment. Correction of pH with 1M HCl was done every day after sampling, small volume (aprox. 5 ml) from flask medium were discarded in 20 ml eppendorf tube, where pH was determined using a pH meter WTW pH 315i. Correction of pH was done by adding 1M HCl or 1M NaOH to experiment flask, applying shaking and repeating withdraw of small volume from flask medium. Correcting cycle was repeated until necessary corresponding pH value was achieved.

Samples were taken at 0, 24, 48, 72, 120 and 168 hour from the start of experiments. In samples total phenol (mg/l), COD (mg/l) for 0.45 µm filtered samples and optical density (OD at 600 nm) were measured; while laccase oxidation enzyme concentration (nkat/ml) was determined in non-filtered samples.

Total phenol and COD was determined using Hach Lange cuvette tests and spectrophotometer DR 5000. TOC was determined using high temperatures Skalar Analytical TOC analyser Formacs HT TOC/TN.

2.6. Laccase Assay

Concentration of laccase enzyme was measured with spectrometer (Camspec M501) at 420 nm. In 1 ml cuvette was mixed 930 µl of pH 5.0 acetic buffer (autoclaved), 50 µl of not filtered sample and 20 µl of 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt e.g. ABTS solution. ABTS solution was made by dissolving 20 mg of ABTS powder in 1 ml of pH 5.0 acetic buffer. After mixing cuvette was placed in spectrometer and optical density decreases due to ABTS oxidation by laccase presence was measured. The time interval of absorbance measurement was 20 seconds. Afterwards absorbance

measurement laccase concentration was expressed in nkat/ml.

3. RESULTS

3.1. Screening Tests

Initial tests were performed to investigate four different fungi species, using various oxidative enzyme systems, about their ability to degrade total phenol. The screening tests were done in synthetic medium A, with starting total phenol concentration 605 mg/l. After determining most effective fungal specie, further optimization essays were carried out. Tests with optimal environment conditions tests were done. As it is evident in Fig. (1), amongst investigated species, *T.Versicolor* was the most efficient, with a removal rate of total phenols of 71% after 7 days of treatment, corresponding to a decrease from 605±18 mg/l to 175±26 mg/l of total phenol concentration. The standard deviation

(SD) increased proportionally with time, indicating degradation trend stability and positive constant removal of total phenol by *T.Versicolor*.

The TOC content for screening tests is given in Fig. (2). TOC reduction potential for *T.Versicolor* was 53% in 7 days, from 9.09 g/l to 4.26 g/l. Most of TOC concentration originated from synthetic medium for boosted growth.

3.2. Optimisation of Water Treatment Parameters

Initially in the tests of optimal environment conditions various amount (v/v) of biomass inoculant was added in the beginning of the experiment. Inoculant added before experiment was grown in sterile medium and homogenised using sterile glass spheres. As it is shown in Fig. (3), adding of biomass concentrations (v/v) 10%, 20% and 30% led to equal removal efficiency of total phenols (71% in 7 days), corresponding to a decrease from 605±18 mg/l to 175±26

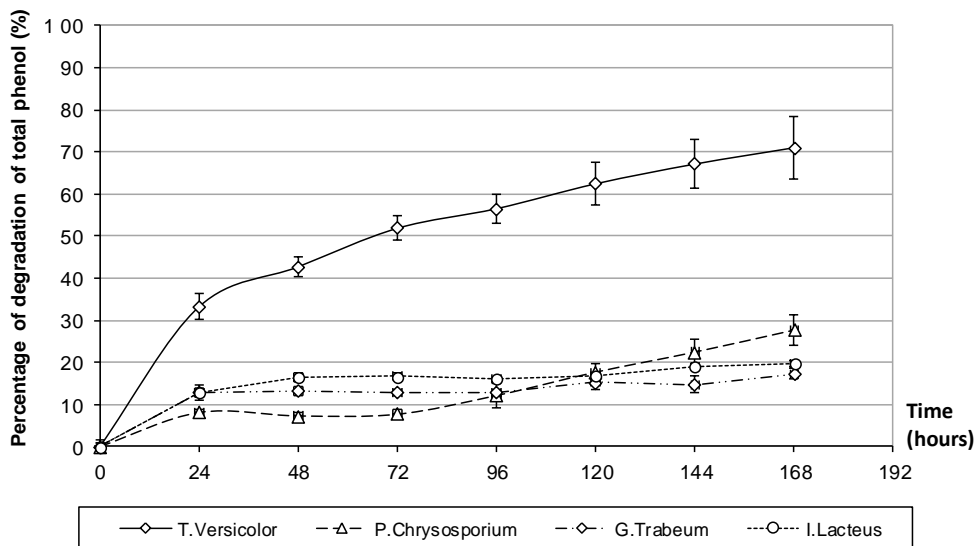


Fig. (1). Phenol degradation screening tests (10% (v/v) inoculum biomass, T=30°C, pH=5.5).

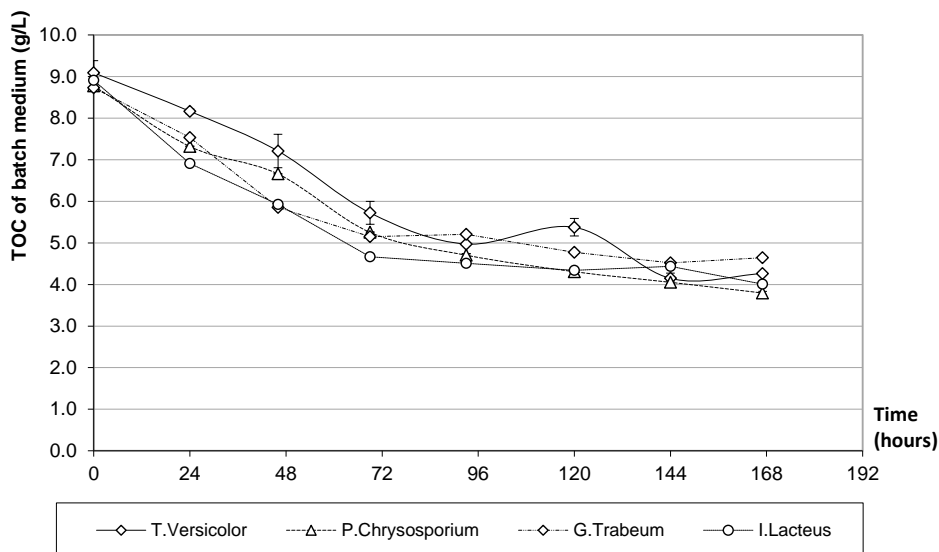


Fig. (2). TOC reducing of phenol degradation screening test (10% (v/v) inoculum biomass, T=30°C, pH=5.5).

mg/l. The only biomass concentration that showed difference was 5%, showing removal of 23% in 7 days, from 597±18 mg/l to 459±53 mg/l. Temperature impact on degradation efficiency is shown in Fig. (4), indicating that most effective medium temperature is 25 °C, removing total phenol by 93% in 7 days, from 420±12 mg/l to 29±1 mg/l. Efficiency of phenol degradation at temperature above 25°C decreased rapidly. The test of medium pH presented in Fig. (5) and Fig. (6) showed that most suitable values for degradation are pH 5 and pH 6, with degradation efficiency 68% and 51% in 7 days. Total phenol removal was from 400 mg/l to 130 mg/l (pH 5) and 210 mg/l (pH 6). The SD for all pH values was low, with 7% being the highest.

The test of medium temperature showed highest SD among all environmental condition tests, SD value of 20 °C for total phenol degradation in day 7 was 46%, being 69±32 mg/l. All other temperatures regimes, above 20 °C, indicated small variation of SD values, being less than 7% of corresponding day concentration. Maximal variation of SD for other condition tests was less, 13% for biomass inoculum tests and 8% for test of medium.

3.3. Correlation Between Parameters

In addition to environmental conditions optimization, the laccase enzyme concentration was monitored for *T.Versicolor*. Results are shown in Fig. (7). Phenol concentration was reduced by 93%, removing from 420±12 mg/l to 29±1 mg/l in 7 days. TOC amount was reduced by 47%, removing from 6.58 g/l to 3.47 g/l in 7 days. Concentration of laccase enzyme being in range from 1.09 (day 1) to 4.53 (day 7) nkat/ml, retaining constant increase during experiment. For *P.Chrysosporium*, *G.Traeum* and *I.Lacteus* species presence of laccase enzyme in screening tests was not detected.

4. DISCUSSIONS

Phenol removal with four different fungi species was established, all species were able to start phenol removal. Only *T.Versicolor* specie in screening test was able to reach the 70% total phenol removal. As reported by other authors, fungal microorganisms were successful for removal of cyclic pollutant in batch scales [19-21]. Optimal pH of medium reported in literature for phenol biodegradation with fungi's

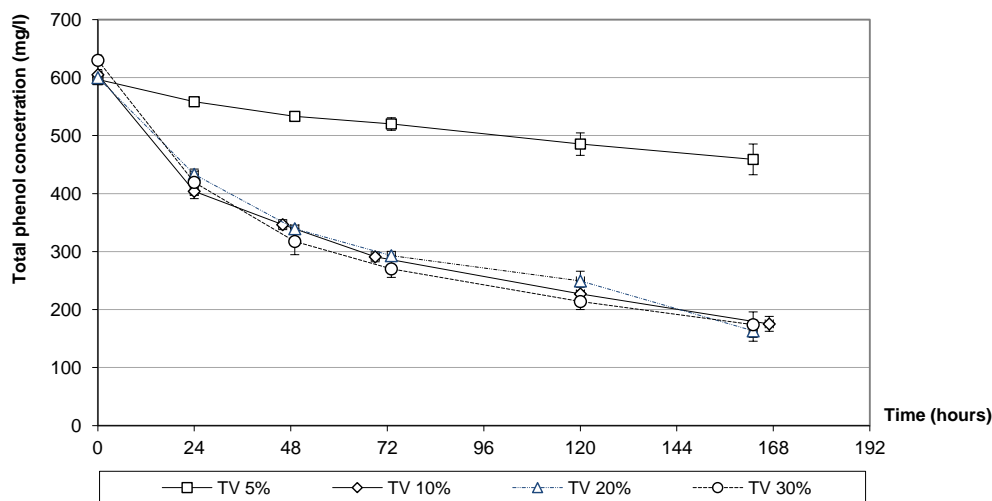


Fig. (3). Inoculum biomass (v/v) influence on phenol degradation (T=30°C, pH=5.5).

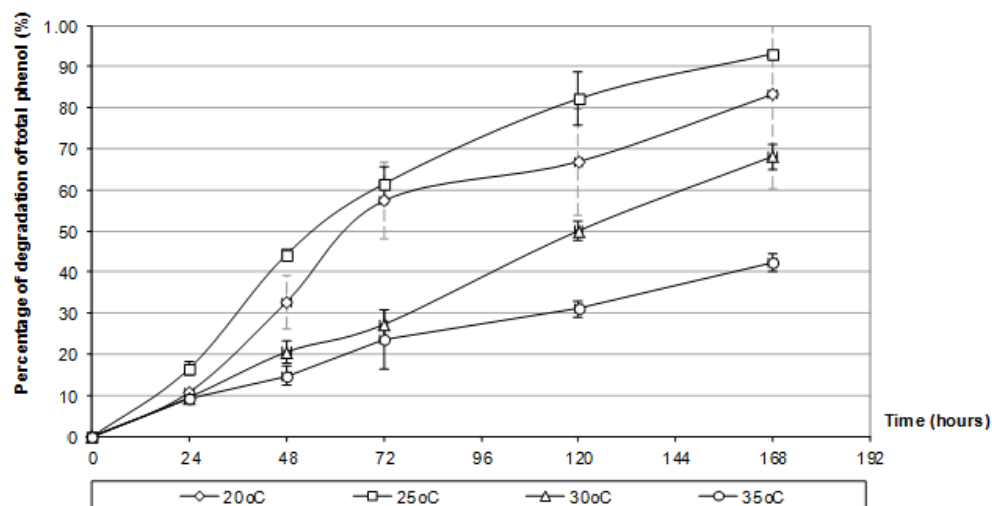


Fig. (4). Temperature of batch medium influence on phenol degradation (10% (v/v) inoculum biomass, pH=5.5).

microorganisms is pH 5-6 [22, 23], which matches with our results. Limiting temperature effect on phenol biodegradation in various fungal bioreactors started from 30 – 34 °C [24], which is in accordance to our results of temperature variations. It was indicated in literature that starting inoculum of fungal biomass is very important and sensitive parameter for further biodegradation rate [25], the concentration inoculum to total volume was in range 3-4 % [26, 27]. In our studies optimal inoculum concentration was concluded 10% of total volume, able to remove phenol with initial concentration of 600 mg/l. Further inoculum increase did not increase phenol removal amount and rate. It is possible that fractions of phenols are moved by biosorption rather than metabolic processes of the cells. However, significant correlation between enzyme activity and removal efficacy of phenols, is indicating that process is dominantly driven by enzymes, which are oxidizing phenols.

The species *P.Chrysosporium*, *G.Traeum* and *I.Lacteus* were able to start removing total phenol in first day by 7 –

13%, but then elimination rate remained constant. For *G.Traeum* and *I.Lacteus* degradation values show process stopping after first day, making minor gradient increase to 19% and 20% till day 7. For this species it is assumed that oxidation enzymes were inhibited by initially degraded phenol, furthermore, enzyme system was able to degrade only linear bounded carbon from synthetic medium. Specie *P.Chrysosporium*, which was reported to be able to perform degradation of cyclic compounds [5], showed minor adaptation. From day 1 to day 3 *P.Chrysosporium* did not show any phenol degradation increase remaining at 7 % constant level, after day 3 showing gradual increase to 28% till day 7. Regarding to this increase for *P.Chrysosporium* showed theoretical ability to make degradation of total phenol.

Specie *T.Versicolor* showed continuous remaining increase of total degradation, indicating that the designed substrate of specie enzymatic system is similar with phenol structure. Combining Fig. (1) with total organic carbon

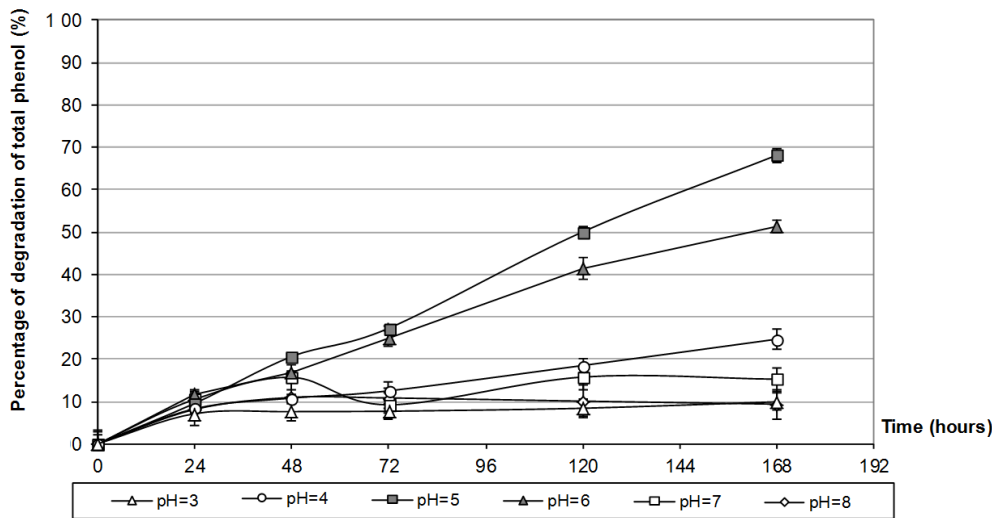


Fig. (5). pH of batch medium influences on phenol degradation (10% (v/v) inoculum biomass, T=25°C)

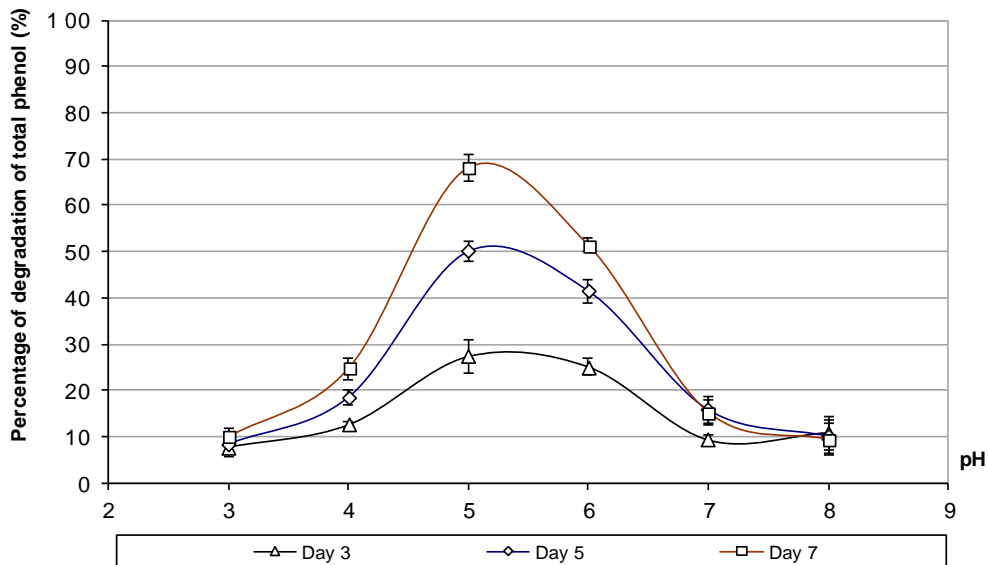


Fig. (6). pH of batch medium influence on phenol degradation (isolines) (10% (v/v) inoculum biomass, T=25°C).

(TOC) reduction results, best phenol degrading specie for first 72 hours has less TOC consumption, since part of carbon is consumed from by total phenol content. Main difference between *T.Versicolor* and other used species is production of oxidative laccase enzyme, which *T.Versicolor* can produce up to 18 nkat/ml [6]. For other species *P.Chrysosporium*, *G.Trabeum* and *I.Lacteus* laccase enzyme was not detected. Because of relatively higher efficiency in by phenol removal *T.Versicolor* as comparing to other used species, for further optimal condition tests this species was used.

4.1. Optimal Conditions

The optimal condition tests showed that the most important criteria for successful phenol degradation with fungi is maintaining pH of medium in range between pH 5 and pH 6, since outside this range phenol removal decreases below removal of 30% in 7 days. Medium pH value affects most rapidly removal rate of all environmental conditions. As well values above pH 6 in raw wastewater will be suitable for bacteria, which will ensure that fungi will be less competitive for nutrients. It can be concluded from Fig. (6) that most effective pH values is located between peaks, which considering curve slopes before peaks is located in range of pH 5.3 – 5.5.

Moreover, biomass concentration of inoculant is one of the most important parameter for phenol degradation. It was found that for total phenol loading rates up to 600 mg/l inoculant biomass concentration of 10% is sufficient and further biomass increase doesn't increase amount of phenol removed. The stock cultures of inoculum, before homogenization, were forming spherical objects from 1 to 10 mm in diameter. Before inoculum addition to biomass test, it was homogenised to value of optical density of 0.300 Abs. The biomass concentration tests were done in sterile synthetic medium, without other microorganisms, which could

compete for nutrients, therefore in raw waste water the required biomass concentration to overcome microbiological inhibitions could be higher.

After environment pH and required biomass inoculation the temperature is the third important parameter that affects degradation process in range of 42 to 93%. Temperature tests indicated that medium temperature above 25 °C notably decreases phenol removing, not promoting good environment for laccase enzyme production and therefore phenol degradation. The best temperature diapason was found between 20 – 25 °C, with increased sensitivity to higher temperatures.

Concentration of laccase enzyme, by maintaining optimal conditions, was gradual increasing all experiment time, as shown in Fig. (7). Our findings support previous research, which showed that basidiomycete fungal monocultures have great ability to degrade phenol, while in this study we demonstrated that incubation conditions impact the biodegradation efficiency.

4.2. Potencial of Fungi Pretreatment Prior Conventional Treatment

The current study was limited to a batch scale and synthetic liquid medium conditions using monocultures in biodegradation tests. Current studies showed that *T.Versicolor* specie whose enzymatic system is capable for phenol degradation can be used for phenol removal from wastewater. However, there are several issues that should be addressed in future research. The study was carried in “sterile conditions”, meaning the experiments were design to exclude other microbes than *T. Versicolor*. In the field conditions there might be competition of fungi with indigenous microorganisms for the nutrients, which could influence overall efficacy of wastewater treatment. This should be addressed by stimulation conditions in which fungi are dominant over bacteria including control of pH and using

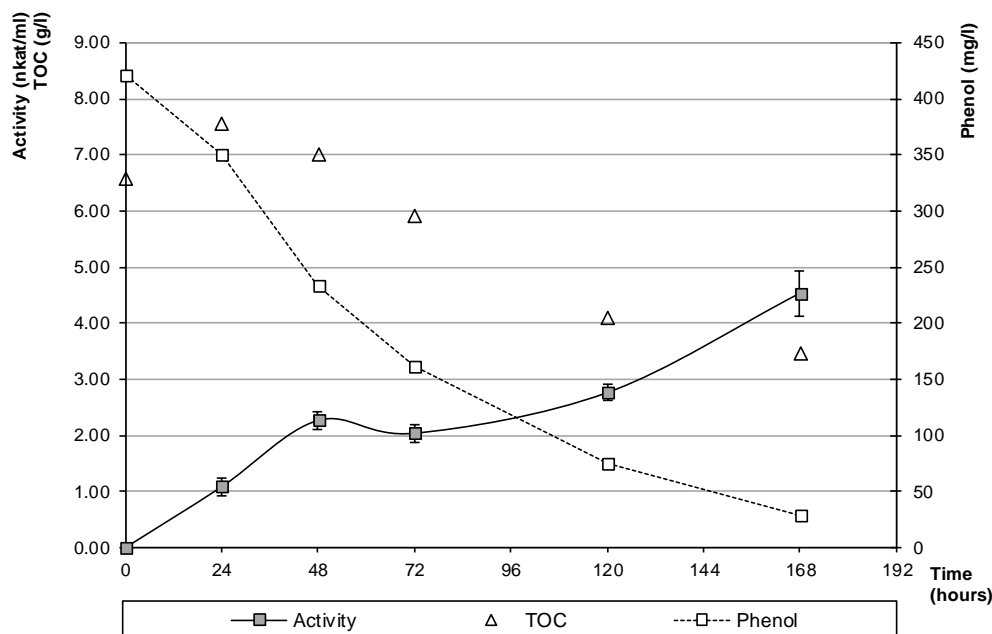


Fig. (7). Degradation of phenol (420 mg/l) with *T.Versicolor* (10% (v/v) inoculum biomass at T=25°C, pH=5.0).

bioaugmentation approach – continuously adding fungi into the reactor.

With all limitations, the proposed technology allows to save energy and reduced waste generation compare to traditionally applied chemical oxidation technologies.

CONCLUSION

The high phenol content in wastewater provides toxicity and inhibition of conventional bacteria bioreactor treatment, leading to limited total phenol removal and effluent toxicity. The batch studies with four microorganism cultures showed that total phenol is removed most effectively by *T. versicolor* specie. The maximum total phenol removal, at optimal environment conditions, was 93% in 7 days, reducing total phenol from 420±12 mg/l to 29±1 mg/l. The optimal conditions for phenol degradation with *T. Versicolor* were found at pH 5-6, temperature 25 °C and biomass mass concentration of inoculum of 10%.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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