Dephenolization of Olive Mill Wastewater by Pellets of Some White Rot Fungi

Bazı Beyaz Çürükçül Fungus Pelletleri ile Zeytin Kara Suyundan Fenol Giderimi

Research Article

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ABSTRACT

The present study was aimed at evaluating the phenol removal efficiency of growing cell of some lignin-degrading fungi. The experiments were carried out both at olive mill wastewater (OMWW) based medium and at OMWW medium supplemented with nutrient with *Trametes versicolor* ATCC200801, *Phanerochaete chrysosporium* ME 446, and *Pleurotus ostreatus*. Correlation could be established between phenol removal determined and extracellular enzyme activity measured end of the incubation time; therefore, enzymatic dephenolization experiments were also performed. With experiments related to growing cell, the most stable strain, optimum incubation time, dilution of OMWW, medium were selected as *T.versicolor* ATCC200801, 9th day, 15%, and only OMWW based medium without nutrient, respectively. In this study, 97% of phenol removal was obtained not including the requirement of adaptation phase, pretreatment, and adding of any nutrients, which these findings, not demonstrated in the previous studies related to OMWW.

Key Words
Olive mill wastewater, Wastewater treatment, Dephenolization, White rot fungi

ÖZET

Bu çalışmada bazı beyaz çürükçül fungusların gelişen hücrelerinin fenol giderim etkinliklerinin değerlendirilmesi amaçlanmıştır. Deneyler, *Trametes versicolor* ATCC200801, *Phanerochaete chrysosporium* ME446 ve *Pleurotus ostreatus* kullanılarak hem zeytin kara suyu ortamında hem de besin içeren zeytin kara suyu ortamında gerçekleştirilmiştir. Belirlenen fenol giderimi ile inkübasyon süresi sonunda ölçülen hücre dışı enzim aktivitesi arasında bir ilişki kurulabilmiştir. Bu nedenle enzimatik fenol giderimi deneyleri de gerçekleştirilmiştir. Hücresel fenol giderimi ile ilgili olarak en uygun suş; *T.versicolor* ATCC200801, optimum inkübasyon süresi; 9 gün, zeytin kara suyu dilüsyonu; %15, yetiştirilen ortam; besin eklemeksizin zeytin kara suyu ortamı olarak seçilmiştir. Bu çalışmada adaptasyon basamağı, ön işlem ve besin eklemeye ihtiyaç duyulmadan %97 fenol giderimi elde edilmiştir. Zeytin kara suyu ile ilgili yapılmış önceki çalışmalarda elde edilen bu bulgulara rastlanılmamıştır.

Anahtar Kelimeler
Zeytin karasuyu, Atık su iyileştirilmesi, Fenol giderimi, Beyaz çürükçül fungus

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INTRODUCTION

The extraction process of olive oil yields a highly polluting byproduct, known as olive mill wastewater (OMWW) creating a major problem in Turkey as other Mediterranean countries. OMWW contains high levels of phytotoxic and antimicrobial compounds due to the fact that it includes monomeric-polymeric phenols, volatile acids, polyalcohols, nitrogenous compounds and has high biological and chemical oxygen demands [1]. This wastewater has been discharged into surface waters and spread to land which affects soil's physical and chemical properties such as porosity and pH. In addition, the high concentration of reducing sugars can stimulate microbial respiration, lowering of dissolved oxygen concentrations, while the high phosphorus content can lead to eutrophication [2].

Because of the complex composition of OMWW, the seasonal nature of olive production and the wide geographical dispersion of mills, there are technical and economic limitations for efficient treatment. The rivers may be affected the high concentration of darkly colored polyphenols in OMWW [2].

Proposed physico-chemical processes such as evaporation ponds or lagoons [3] have not been efficient in decreasing the high COD, toxicity of OMWW. To reduce the ecological impact of OMWW, biological remediation is an alternative to these physico-chemical processes which are commonly unfeasible technically and economically. In general, fungal remediation of OMWW has been studied using lignin degrading organisms known as white rot fungi having an ability of removal of phenolic substances which are lignin-like structure and recalcitrant compounds. They have large amount of potential for the biodegradation of polycyclic aromatic hydrocarbons [4], pesticides and other pollutant compounds such as cyanide [5,6], decolorization of reactive dyestuffs [7,8], dechlorination of chlorophenolic compounds [9], solubilization [10] and desulphurization of coal [11,12] through their biomasses or extracellular enzymes including laccase, manganese-dependent peroxidase and lignin peroxidase produced by these fungi.

Several white rot fungi including *Trametes versicolor* and *Funalia trogii* [13,14], *Lentinula edodes* [15], *Phanerochaete* sp. [16], *Pycnoporus coccineus* [17], *Coriolopsis polyzona* [18], *Pleurotus ostreatus* [19], species of *Cerrena, Byssochlamys, Lasiodiplodia* and *Bionectria* [20] have been used for remediation of OMWW.

Effective bioremediation resulting in significant reduction of phenolics allows safe and economical disposal of OMWW onto land or into surface waters. As another benefit, bioremediation may produce valuable products including an excellent fertilizer [3].

The purpose of the present work is to investigate the abilities in reduction of phenolic compounds in OMWW of some white rot fungi such as *Trametes versicolor, Phanerochaete chrysosporium* and *Pleurotus ostreatus* through growing cells and their enzymes.

MATERIALS AND METHODS

Characteristics of OMWW

The experiments were performed with OMWW provided from a two-phase plant (Edremit of Turkey), exhibiting the following characteristics: pH, 4.8; chemical oxygen demand (COD), 133.075 g/L; total phenols 5.105 g/L. OMWW was centrifuged (9000 x g, 20 min), filter-sterilized and stored at -20°C until usage.

Growing cell studies

Phenol removal abilities of microorganisms containing *Trametes versicolor* ATCC 200801, *Phanerochaete chrysosporium* ME 446 and *Pleurotus ostreatus* were tested during growth under agitated culture conditions. *P. ostreatus* was obtained from Dr. I.F Zadrazil, FAL Braunschweig, *P. chrysosporium* ME 446 was taken from Dr. T.K. Kirk (U.S. Department of Agriculture Forest Products Lab., USA). After preculture incubation at 30 °C on Malt broth (Merck) for 4 days, obtained fungal pellets were weighed at equal amounts (0.3% w/v) and used as inoculum. Pellets were transferred into 100 mL liquid medium in a 250 mL flask for agitated culture conditions (150 r.p.m.). Media used for phenol removal were only diluted OMWW and malt broth liquid medium which is

based on diluted olive mill wastewater at different dilutions. All cultures were incubated at 30°C for 10 days and spectrophotometric measurements of phenol removals were measured everyday.

For evaluating the effect of biomass amount on dephenolization with growing cell, different amount (0.1-5% w/v) of biomass was inoculated to medium only diluted OMWW medium without supplement. Furthermore, the experiment was performed to determinate the tolerance of OMWW dilution on phenol removal of OMWW with growing cell by diluting OMWW the rate of 15, 25, 35, 50, 75% and undiluted.

Non-inoculated controls were incubated in parallel under the same conditions. All data were the means of three different replicates.

Enzyme studies

After growing cell studies, thought that is responsible for phenol removal, the extracellular enzyme which excreted the examined fungus, was evaluated at the study. At the result of biomass studies, the efficient strain at phenol removal was selected *T. versicolor* ATCC200801 and this strain was grown on submerged cultures in potato dextrose broth (PDB) using wheat bran as ligninolytic enzyme inducer, according to Gedikli [21]. After 12 days of culture, supernatant was filtered and used as crude laccase source at all dephenolization experiments with enzyme whose activity was 22 U/mL. The reaction mixtures for phenol removal activity were in 10 mL working volume in static tubes in water bath (Memmert). Incubations with heat-denatured laccase served as a control under the same incubation conditions. The laccase-catalyzed OMWW dephenolization was measured in a cuvette of the spectrophotometer (Schimadzu UV-Vis Spectrophotometer 2550, Tokyo, Japan).

pH parameter was studied at OMWW dephenolization with enzyme owing to the fact that providing the stability properties of enzyme to for continuity of treatment is important. Tubes containing OMWW (15%) were adjusted at varying pH (pH 3-12 and not adjusted pH) using at 37°C, enzyme amount of 0.2 mL and reaction time of 30 minutes. For pH 3-5, sodium acetate buffer adjusted pH with acetic acid; for pH 6-8, Na_2HPO_4-NaH_2PO_4 buffer and for 9-12, $NaHCO_3$-NaOH buffer were used. OMWW dilutions were prepared with corresponding buffer solutions excluding not adjusted pH whose dilution was prepared with dilute water. At the end of the reaction time, phenol measurement was performed. The denatured enzyme and OMWW was used by making constant other conditions as control group.

To investigate the tolerance of the laccase against the OMWW, the dephenolization was carried out with varying dilutions (15-25-35-50-75%). This experiment was run at 37°C, 0.2 mL of enzyme amount for 30 minutes, pH 4.5 which was found as optimum pH.

In order to examine the effect of reaction time on enzymatic dephenolization, 1-3-5-15-30-45-60 minutes and 2-6-12-24-36-48-72-96 hours were studied at this experiment. Parameters defined before were constant.

The experiments related to enzyme amount were achieved with different amounts of enzyme (0.05-3 mL). Other parameters were constant including 37°C, 25% dilution of OMWW, 30 minutes, pH 4.5 which was found as optimum pH.

Catalase treatment

Although the culture supernatant contained high laccase activity, the culture supernatant may also contain peroxidases, which was tested with catalase (Sigma) treatment.

Analytical assays

Total phenol was determined by the Folin-Ciocalteau reagent with gallic acid as the standard [22]. Concentration of COD was determined according to APHA [23]. Reducing sugar was assayed by the Somogyi and Nelson method, using glucose as standard [24]. Laccase activity was assayed according to the described protocol [25]. To determinate enzyme activity, 0.1 mL enzyme source was added to 4.9 mL of 0.1 M sodium acetate buffer (pH 4.6) containing 1 mM guaiacol as the substrate. The reaction mixture prepared was incubated at 37°C for 15 minutes. Enzyme activity in the tubes was measured by reading optical density in the UV-

Visible spectrophotometer adjusted to 465 nm wavelength. (Schimadzu UV-2550). 1 U of enzyme activity was defined as the amount of enzyme that elicited an increase in A465 of 0.1 absorbance unit per minute. Incubations with denatured laccase served as a control.

Bacterial toxicity of the OMWW after biological treatment

Toxicity tests for Bacillus subtilis (NCIB 3610) were performed variably diluted (15% and 50%) OMWW treated by growing cell of *T. versicolor* for 9 days, the most effective day at phenol removal in our study. These experiments were conducted in the presence of untreated OMWW (controls) which were also run in parallel.

Discs with 6 mm of diameter were sterilized. The samples to be tested were previously filtered through 0.22 mm filters under sterile conditions and adsorbed 50 μL to each discs on the surface of nutrient agar plates spreading with 106 colony-forming units (CFU)/mL of cell suspension were equal to the turbidity of a 0.5 McFarland standard. After 48 h of incubation at 37°C, the plates were examined for halos of inhibited growth around the discs and measured diameter of zone by compass.

RESULTS

Choose of strain, medium and incubation time to biologically treat OMWW

The abilities of three fungi in removing phenol were tested using OMWW without the addition of any nutrients. As shown in Figure 1, phenol removal reached about 97% by the 9th day of incubation for OMWW with 15% dilution. *T.versicolor* was observed to give more stable and higher results than other fungi tested in both media, which indicated that the range of polyphenol concentration examined was tolerated and substrate inhibition was negligible especially at low phenol concentrations; therefore, this organism was selected for further experiments. In results related to *P.chrysosporium,* phenol removal from OMWW was especially detected at 15% and 25% dilutions of OMWW and started on 6th day following adaptation to medium. In the matter of optimum day, when the amount of OMWW was enhanced in medium, it seemed to extend the day of maximum dephenolization among the dilutions assessed. 9th day was chosen as most favorable day because this day was found maximum dephenolization as short as possible.

As shown in Figure 2, the fungus growing the OMWW medium supplied malt broth including nutrients removed more phenol than OMWW based medium on the first days. Only OMWW based medium without any nutrients was chosen among two media investigated because of its being more economical.

Figure 3 shows the effect of biomass amount on phenol removal of OMWW with growing cell having *T.versicolor*. 5 g of biomass amount was chosen for further experiment related to tolerance to initial polyphenol concentration of *T.versicolor* growing cell.

According to our results obtained, 14.43% of dephenolization with 5 g of *T.versicolor* pellets at the end of 9 days was determined at undiluted OMWW in the absence of external organic supplements without spending adaptation period.

However, dilutions of OMWW with water were prepared to assist fungus growth in wastewater and phenol removal which reached 97.90% by diluting 15% of OMWW (Figure 4).

Enzyme treatment

Enzymatic treatment has the advantage of a wastewater treatment with shorter time than fungal cultures. Therefore, we investigated culture supernatant with high laccase activity as enzyme source in respect of phenol removal.

In a previous stage of the work related to growing cell, it was determined the best ability of *T.versicolor* among three strains examined to remove phenol from OMWW.

The extended incubation is assumed to result in accumulation of laccase, permitting rapid dephenolization. Thereby, we investigated ligninolytic enzymatic system thought that involved in the process by inducing activity of enzyme.

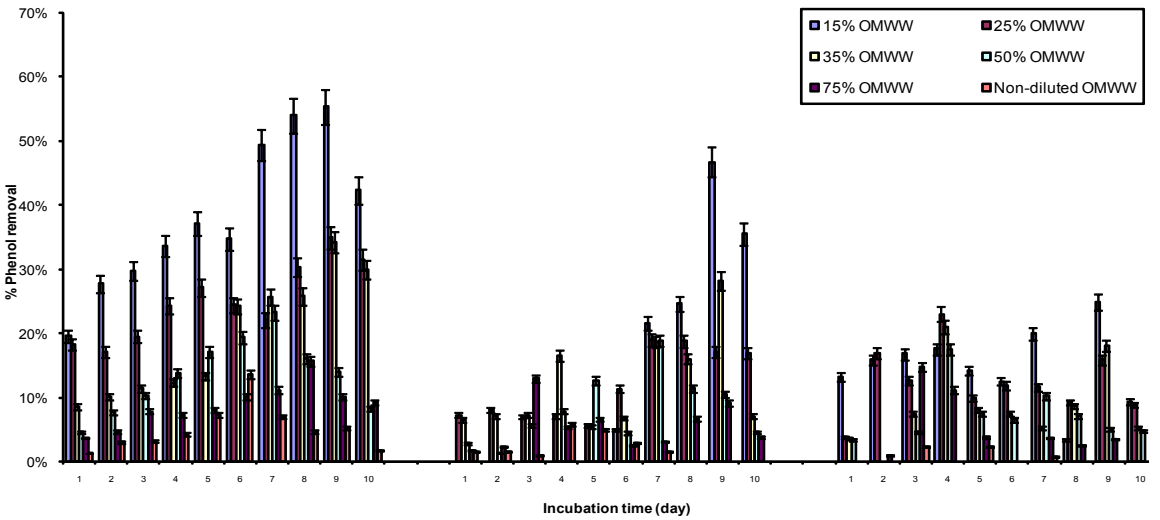

Figure 1. Daily phenol removal in olive mill waste water (only OMWW diluted with water) with growing cell having *T.versicolor, P.chrysospsorium* and *P.ostreatus* (Working conditions: 30°C, 150 r.p.m., 0.3% w/v)
Note: 1st,2nd,3th groups in figure refer *T.versicolor, P.chrysosporium, P.ostreatus*, respectively. Error bars represent standard error.

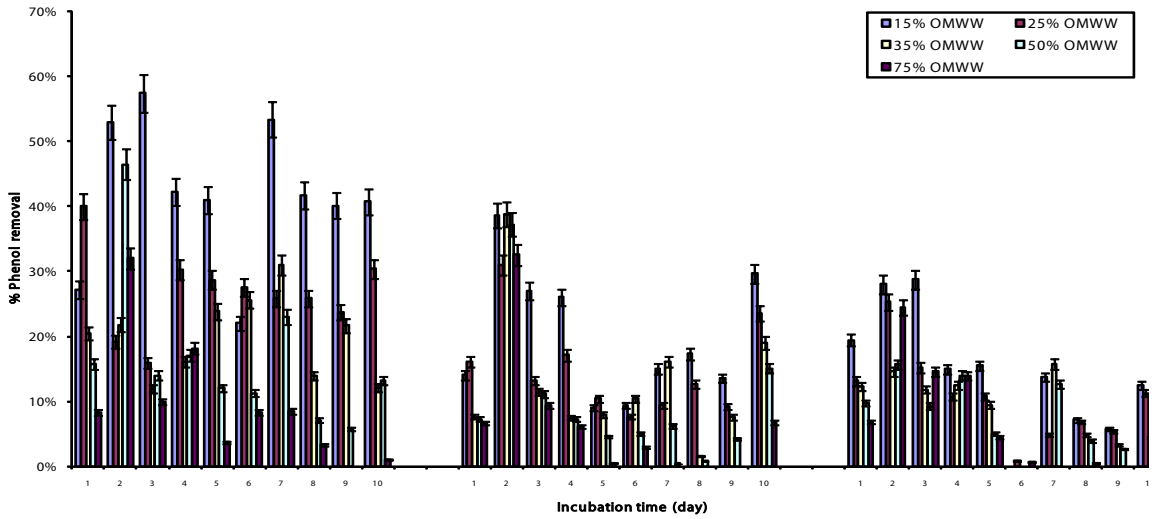

Figure 2. Daily phenol removal in olive mill waste water (OMWW diluted with malt broth medium) with growing cell having *T.versicolor, P.chrysospsorium* and *P.ostreatus* (Working conditions: 30°C, 150 r.p.m, 0.3% w/v)
Note: 1st,2nd,3th groups in figure refer *T.versicolor, P.chrysosporium, P.ostreatus*, respectively. Error bars represent standard error.

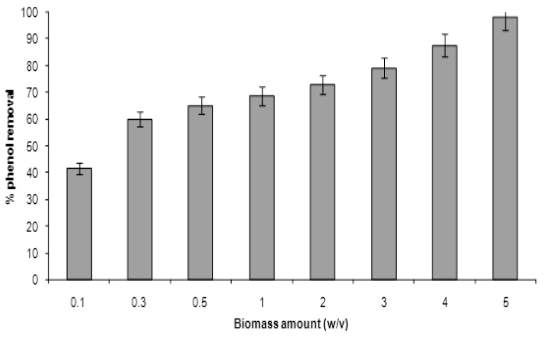

Figure 3. Effect of biomass amount on phenol removal of OMWW with growing cell having *T.versicolor* (Experiment conditions: 15% diluted of OMWW without supplement, 30°C, 150 r.p.m., 9 days of incubation time, 100 mL of working volume). Error bars represent standard error.

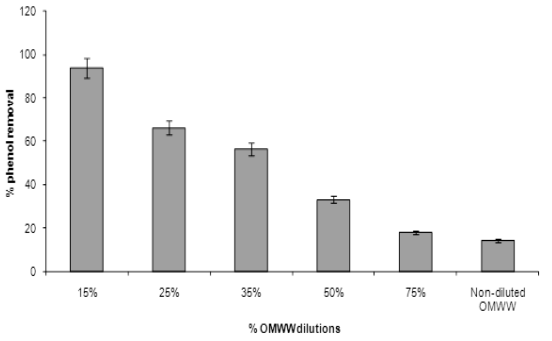

Figure 4. Tolerance of OMWW dilution on phenol removal of OMWW with growing cell having *T.versicolor* (Experiment conditions: 5 g of biomass amount, 30°C, 150 r.p.m., 9 days of incubation time, 100 mL of working volume). Error bars represent standard error.

Because of the fact that it is essential to offer pH stability in all enzymatic studies, pH value of reaction medium was investigated in enzymatic dephenolization of OMWW examined. In pH optimization experiments, pH 4.5 was found as the best suitable pH for the reactions giving maximum yield of dephenolization (Figure 5).

As a result of the experiment performed to find out optimum reaction pH for enzymatic removal of phenolics, the maximum percentage of dephenolization was at ratio of 16.452%. The results related to optimization of initial phenol concentration are plotted in Figure 6.

Initial phenol concentration examined was chosen 25% diluted OMWW. Concentration of phenol which is a toxic compound was tolerated by laccase by this value. The percentage of dephenolization at this phenol concentration was found 16.969%.

The effect of reaction time on enzymatic phenol removal was investigated under static conditions.

As shown in Figure 7, optimum reaction time was found as 2880 min with 28.954% of dephenolization.

To determinate the influence of enzyme amount on enzymatic dephenolization, experiments were conducted at the range of 0.05-3 mL.

As it can be seen in Figure 8, when enzyme amount was enhanced, rate of enzymatic dephenolization increased.

The changes occurring at treated OMWW

With biological treatment, changes occurring at OMWW were determined (Table 1). 15% diluted of OMWW incubated with *T.versicolor* for 9 days and observed the best extent of dephenolization at preliminary studies. Under these conditions, it was compared in respect of total phenolic removal as well as reducing sugar, consumption of COD, laccase activity obtained before and after the treatment. The experiments were conducted in a batch system. The control group was 15% diluted OMWW untreated.

Table 1. Changes occurring at OMWW treated (15% diluted of OMWW treated with 5 g of *T.versicolor* growing cell for 9 days comparison of 15% diluted of OMWW untreated)

Medium	Total phenol removal (%)	pH	Reducing sugar consumption (%)	Laccase activity (U mL^{-1})	COD removed (%)
OMWW treated	97.90	5.4	17.26	1.941	13.59

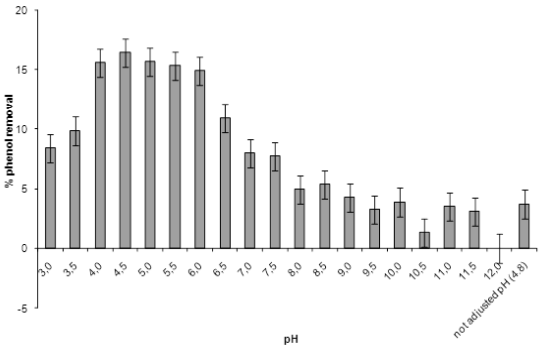

Figure 5. Effect of medium pH on dephenolization of OMWW with crude laccase (Working conditions: 37°C, static mode, 0.2 mL /10 mL v/v, 15 minutes, 15% diluted of OMWW with buffer solution but no supplement)
Note: OMWW without adjusting pH was prepared with diluted water. Error bars represent standard error.

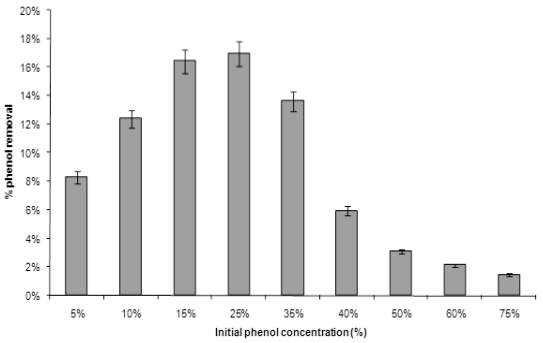

Figure 6. Effect of initial phenol concentration on dephenolization of OMWW with crude laccase (Working conditions: 37°C, static mode, 0.2 mL/10 mL v/v, 15 minutes, pH:4.5). Error bars represent standard error.

Bacterial toxicity of the OMWW after biological treatment

The effect of biological treatment of OMWW on the bacterial toxicity was observed after cultivation for 48 h, using *B. subtilis* (NCIB 3610) as a sensitive test strain.

The levels of bacterial toxicity naturally present in untreated OMWW decreased concurrently with removal of phenolic compounds. Inhibition zone surrounding the discs absorbed 15% and 50% of OMWW untreated were 6.57 and 9.81 mm, respectively; that of treated were 6.23 and 7.56 mm, respectively.

DISCUSSION

In the study of dephenolization with growing cell, the strain, incubation period, dilution rate, the amount of biomass, and the medium were selected *T.versicolor*, 9 days, 15% of dilution rate, 5% w/v of biomass, and the medium with only OMWW, respectively (Figures 1-4). Figure 2 showed that addition of malt broth in OMWW did not enhanced phenol removal by the fungi examined in certain cultivation. Hence, dephenolization of OMWW, the performance of *T.versicolor* with only diluted OMWW in working conditions was evaluated. Using of supplement with malt broth might support only growing the fungal cell but cause no significant effect on the dephenolization process. Therefore, utilizing of OMWW diluted with water was preferentially studied and 9 days of incubation period was chosen as optimal value in this study. Olivieri et al. studied OMWW remediation with *P. ostreatus* and observed that polyphenols abatement was controlled by the availability of the nutrients [19]. These results are in agreement with our results, and there was a balance in respect of phenol among the strains and dilutions investigated. Because the change in wastewater characteristics was estimated; hence, increase and decrease at dephenolization day by day was possible. We predicted that chemical oxidation and sudden variations in the composition of the OMWW might be as well as biological degradation during the incubation time as well; probably, it was a reason of fluctuation at Figure 2, *T. versicolor* was observed to reduce phenol removal in this medium, especially on the 5^{th} and 6^{th} day; but, again increase was detected on the 7^{th} day of incubation, perhaps it might release linked phenols in the presence of nutrients at this day.

OMWW treatment by ligninolytic fungi has been previously reported by numerous authors. It was not easy to compare incubation time required, the percentage of the phenolics removed, and the compositions of the media, on account of complex structure of OMWW and the difference in initial phenol concentrations. Some researchers utilized as an OMWW culture medium without additional nutrients, but OMWW used had low total phenolic content [26,27]. Most studies were focused on growing fungus in synthetic media supplemented with carbon and the other components including different amounts of diluted wastewater or

diluted OMWW based medium supplemented by some nutrients at OMWW treatment [14, 17, 28-30].

In our study, growing cells of *T. versicolor* ATCC200801 without adaptation have been grown in undiluted OMWW medium. Only use of the 4 days of preculture incubation facilitated rapid establishment of fungal species in OMWW and resulted in considerably improved removal of phenolic compounds in this study. Ergül and coworkers investigated possibility of reducing the phenolic content without dilution and without any addition of nutrients or pretreatment by using the white-rot fungi *T.versicolor* FPRL 28A INI [13]. However, they adapted fungus to grow in all concentrations of OMWW and perform dephenolization.

According to enzyme studies, the effects of pH, initial phenol concentration, reaction time, and enzyme amount on dephenolization of OMWW were investigated. A reason for observing phenol removal at pH value of OMWW's own not adjusted with buffer solutions may be close to optimum pH of dephenolization with enzyme.

However, trials at pH values higher than 6.5 are negligible, since *T. versicolor* laccase activity is low at these pHs; moreover, at pH higher than 7.5, auto-oxidation phenomena of phenol may be relevant. Therefore, it is rational that the dephenolization experiments have been performed at the limited pH range characterizing OMWW (i.e, 3.5-6). In case of pH experiment; consequently, pH 4.5 was found as the best value for dephenolization (Figure 5). This value also is known as optimum pH for laccase activity [31].

Initial phenol concentration examined was chosen 25% diluted OMWW and after this value, phenol removal process was considerably reduced (Figure 6). The percentage of dephenolization at this phenol concentration was found 16.969%. Fenice et al. studied laccase of *Panus tigrinus* in OMWW-based medium and observed that any reaction did not occur when the phenol content in the medium was above 1.9 g/L [32].

The effect of reaction time on enzymatic dephenolization was examined and optimum reaction time was found as 2880 min (Figure 7). In the study performed to show the influence of enzyme amount on enzymatic phenol removal, when enzyme amount was enhanced, the rate of enzymatic dephenolization increased (Figure 8).

Jaouani and coworkers studied also degradation of OMWW fractions by *P. coccineus* laccase in absence and presence ABTS and suggested that the treatment of OMWW with the laccase showed similar results to those reported with the fungus indicating that laccase plays an important role in the degradation process [17]. However, after treatment of OMWW with growing fungal cell, low laccase production (1.941 U/mL) in this toxic wastewater was observed at 9th day of incubation in our study. D'Annibale et al. studied the removal efficiency of phenolics in OMWW

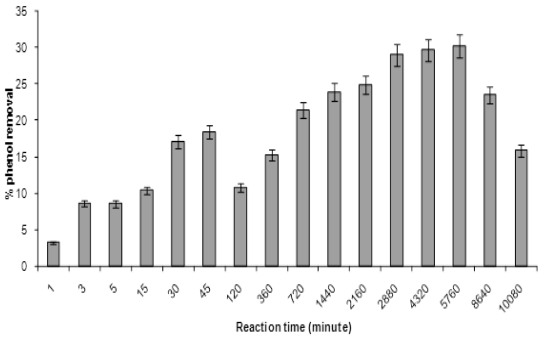

Figure 7. Effect of reaction time of enzyme on dephenolization of OMWW with crude laccase (Working conditions: 37°C, static mode, 0.2 mL/10 mL v/v, 25% diluted OMWW, pH:4.5).

Error bars represent standard error.

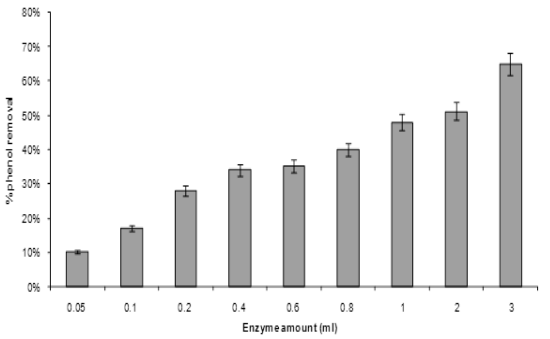

Figure 8. Effect of enzyme amount on dephenolization of OMWW with crude laccase (Working conditions: 37°C, static mode, total volume of 10 mL, 25% diluted OMWW, 2880 minutes, pH:4.5).

Error bars represent standard error.

with immobilized *Lentinula edodes* laccase and reported also parallel data [33]. Our results of enzymatic experiments are also in agreement with those obtained by the growing *T.versicolor* pellets. According to these conclusions, laccase activity was detected at all dilutions of OMWW. However, Jaouani et al. showed that the peaks of polymerized products were lower than the ones found in the treatments without mediator and thought that the mediator may prevent the repolymerization of degradation products besides participating in the degradation of non-phenolic compounds [17].

Indeed, the well known degradation products of OMWW, caffeic acid, catechol, 3,4-dihydroxy-phenylethanol and other aromatic compounds are inducers of laccase [33].

In our enzymatic step at dephenolization, the increase of color was observed. The cause of event may be due to the oxidation of phenolic compounds, possible inducers or substrates of laccase [34].

Casa et al. reduced significantly OMWW total phenol content with fungal laccase treatment, and this enzyme affected preferentially on ortho-diphenolic structures [35]. However, veratric acid and 3,4,5-trimethoxybenzoic acid were also degraded although they were not substrates accepted of the enzyme, on account of the lack of appropriate substituents on the aromatic ring allowing the enzyme action which could be owing to their copolymerization and entrapment within the growing phenolic polymer chain. It was supposed that an increase in OMWW coloration might result from the polycondensation of phenolics to give more colored compounds [36]. We also showed similar results due to the fact that it was probably enzymatic oxidation of phenolics in OMWW.

The experiment of catalase treatment showed that the culture supernatant contained high laccase activity was responsible for the phenol removal. Thereby other enzymes such as peroxidases may not play role in this process.

The changes occurring at treated OMWW was shown at Table 1. Increase of laccase and activity in OMWW based medium may be a metabolic response by the fungus to chemical stress for overcoming the toxic effects of some constituents of wastewater [34]. This defense mechanism occurred in the presence of phenolics aimed at converting simple phenols into reaction products which can not penetrate into the cells by stimulating these enzymes [1] In similar studies, enhancement of phenol oxidase activity in medium including OMWW was also determined white rot fungi such as *Lentinus edodes* [37], *P.ostreatus* [38], *Panus tigrinus* [39], and *Funalia trogii, Pleurotus sajor-caju* [40]. Also, after biological treatment, decreasing of reducing sugar, increasing pH, and consumption of COD were observed. These results show that contamination of soil and aquatic system with OMWW may be decreased. Especially, increasing of final pH (changing from 4.8 to 5.4 in our study) of the effluent after biological treatment was a significant result because reduction of acidity of treated OMWW should be an advantageous target to achieve before release of the waste in the environment. Such a successful biotreatment in respect of an environmentally friendly wastewater, in particular characterized by a reduction of acidity was achieved Alaoui et al. [41].

Indeed, an evident showing the occurrence of chromophore adsorption, a gradually darkening of the mycelial pellets was observed in this study with growing cell. A similar pattern was also reported D'Annibale et al. [39]. However, effective decolorization was not observed in our study. Phenomenon of sequestration of phenolic fragments exerted by the mycelium has been suggested to be an important result of determining decolorization [16,42]. To improve decolorization efficiency, addition of different organic supplement such as sucrose, yeast extract and glucose to OMWW and prolonged incubation time may be required. This necessity, adding of carbon and nitrogen sources supporting wastewater decolorization by white rot fungi, was also suggested in other studies [29,37]. Massadeh and Modallal observed that color reduction started on the 10th day while most of the phenolics were degraded at that time (43). On the other hand, contribution of ligninolytic enzymes such as laccase to OMWW decolorization is still being discussed; even connection was not

found between color removal and laccase and manganese dependent peroxidase production [28]; though some researchers found a correlation between enzyme production and color removal [29]. Sayadi and Ellouz reported to require LiP for OMWW decolorization [42]. Similarly, Jaouani et al. obtained better decolorization where LiP was produced suggesting a predominant role of this enzyme in OMWW decolorization [18]. However, LiP activity was not determined in our study. Probably, the effective production of LiP by changing or/and modifying medium used and extending incubation period may contribute decolorization of OMWW.

The results of bacterial toxicity obtained exhibited parallelism with results reported for antimicrobial activity of OMWW before and after biological treatment [44]. In our study, bacterial toxicity naturally present in untreated OMWW reduced concomitantly with removal of phenolic compounds with *T.versicolor* pellets. Kissi et al. reported an outstanding reduction of the OMWW toxicity on *Bacillus cereus*, after biotreatment with *P. chrysosporium* [45]. Martirani and coworkers reported that decrease in its toxicity was not observed when tested on *Bacillus cereus* 6E:2 although the treatment of olive mill wastewater with laccase excreted the white-rot fungus *P. ostreatus* provided a significant reduction of the phenolic content in the effluent [38]. They suggested that the reaction products, oxidative coupling polymers, quinonoid structures, can be more inhibiting characteristics than the original substances, untreated biologically. Capasso and coworkers also found that ortho-benzoquinone, which can be generated by the laccase-catalyzed oxidation of catechol, completely inhibited the growth of *Pseudomonas syringe* and *Corynebacterium michiganense* at 500 mM concentration [46]. These differences may be owing to a dissimilar sensitivity of the microorganisms tested and different chemical composition of the degradation products of effluent after biological treatment.

CONCLUSIONS

According to the results from this study, white rot fungi strains examined were able to grow in OMWW without any addition of nutrients and adaptation and in this way, remove a major part of phenolic compounds. Enzyme activity measured underlined the suggestion that laccase plays a key role in the dephenolization of OMWW. However, when comparing laccase excreted from *T.versicolor*, using of growing cell of *T.versicolor* at dephenolization might be more advantageous.

The next stage of this study will focus design and scale-up a reactor system. Proposed biological process of OMWW treatment is the combination of physicochemical process and membrane filtration. Besides, the immobilization, using of different inducers and mediators in respect of dephenolization potential for laccase produced by *T.versicolor* are under investigation.

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