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Decolourisation and detoxification in the fungal treatment of textile wastewaters from dyeing processes

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Abstract: In this study a selected fungal strain, *Bjerkandera adusta* (Willdenow) P. Karsten MUT 3060, was tested in different culture conditions in order to assess its real potential for bioremediation of textile wastewaters in terms of both decolourisation and detoxification. The fungus efficiently decolourised (colour removal up to 96%) four simulated wastewaters that mimic the recalcitrance of real ones for pH values, concentration of dyes, additives and salts. In the culture condition with the lowest N content, the decolourisation was coupled with an important detoxification of two simulated effluents, underlining the important influence of the cultural medium composition not only on the degradation but also on the detoxification of industrial wastes. In the other cases, despite an extensive decolourisation, no detoxification was observed. The fungus was further tested against a real effluent, collected from a wastewater treatment plant before and after the tertiary treatment (ozonation) in order to compare the two technologies in terms of chemical and toxicological parameters. The fungal treatment, although less efficient than ozonation, caused a good decolourisation of the effluent, with colour values within the threshold limits of the Italian law; both the fungal and the ozone treatment caused a detoxification, but only towards one of the three organisms used for the ecotoxicological tests. These results underline the critical importance of the ecotoxicological analysis in assessing the applicability of a wastewater treatment.

Turin, 23th June 2011

Dear Dr. P. Lens,
Special Issue Guest Editor of
New Biotechnology

We thank you for your consideration and for the comments and suggestions provided by the reviewers.

In attach please find a revised version of the paper entitled “Decolourisation and detoxification in the fungal treatment of textile wastewaters from dyeing processes”, manuscript number: NBT-D-11-00033.

We modified the manuscript according to reviewers’ comments. In attach you will find the answers to the comments of the reviewers together with an indication of the parts of the text that have been modified (highlighted in the text).

Thank you very much in advance.

Sincerely yours
Dr. Giovanna Cristina Varese

Answer to reviewers

We thank you for your review, comments and suggestions. We would like to respond with the following:

Reviewer 1

- 1) Material and methods. The hydrolysis protocol, the individual dyes concentrations and all the information concerning the real wastewater were added. We also propose to add the absorbance spectrum of the real effluent before and after fungal/ozon treatment (Figure 5). In case Figures were too many, Figure 2 could be omitted. We explained in more detail the calculation of the decolourisation percentage.
- 2) Results. Table 1 was divided in two: Table 1 with enzymatic activities and Table 2 with ecotoxicity tests. We inserted the standard deviations or the confidence limits for the data presented. As we explained in Material and Methods, the toxicological (*V. fischeri* and *P. subcapitata*) and COD analysis of the real effluent were conducted at ARPA Piemonte (the Italian environmental protection agency) that, in accordance with its internal procedures, does not provide confidence limits when the sample is within the terms of the law. However, in our opinion, there still seems appropriate to present the data because they highlight a trend of increased toxicity following both treatments.
- 3) All the Figures were improved according to the reviewer comments.
- 4) A better comparison between the present results and other articles was added.

Reviewer 2

- i) The title was modified in “Decolourisation and detoxification in the fungal treatment of textile wastewaters from dyeing processes”.
- ii) The introduction was modified according to the reviewer suggestions. Four reviews were cited and new comments were added on the choice of enzymatic assays and of the organisms for eco-toxicity analysis.
- iii) Material and methods were modified according to the given indications.
- iv) As explained in Material and Methods, the eco-toxicological tests were conducted on samples and on non-toxic controls containing water (*C. sativus*) or nutritional/diluent solutions (*P. subcapitata/V. fischeri*). Control data are included in the formulae used for toxicity calculation and hence, the measures of toxicity before and after fungal treatment are all relative data that take into account the growth of the organism in the test samples compared to the non-toxic controls. The absolute measurement of growth in the controls (data not shown) has no meaning in itself, but merely serves as a reference for the data obtained on the toxic samples and as proof of the validity of the test. Similarly, the control datum for the DP is included in the calculation of the percentage of color removal compared to the abiotic control, as explained in Material and Methods. We added in the text information of DP values observed in killed samples.
- v) As regard to the enzymes analysis, we performed activity assays in order to investigate the production of peroxidases and laccase that, according to literature, are considered the main involved enzymes in dyes degradation by fungi. The measure of high levels of activity of a specific enzyme, together with the removal of a pollutant, is considered indicative of a relation between the detected enzyme and the degradation. We added a comment on this correlation in the text. However, as we stated, “further studies are necessary in order to identify more precisely these enzymes and to clarify their role in the decolourisation process”. We removed the term “isoform” and corrected with “enzymatic pattern” and, as we have no explanation of the assertion “It could be

assumed that the initial adsorption step ... dye degradation”, we removed it. In the introduction we added, as an explanation of the choice, that the organisms used for ecotoxicity tests showed a good or high sensitivity to the toxicity of textile wastewaters (Tigini et al., 2011).

- vi) Figure caption 2 was corrected and figures were modified as suggested.
- vii) We corrected English throughout the text according to the reviewer suggestions.

**Decolourisation and detoxification in the fungal treatment of textile wastewaters from dyeing
processes**

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Abstract

In this study a selected fungal strain, *Bjerkandera adusta* (Willdenow) P. Karsten MUT 3060, was tested in different culture conditions in order to assess its real potential for bioremediation of textile wastewaters in terms of both decolourisation and detoxification. The fungus efficiently decolourised (colour removal up to 96%) four simulated wastewaters that mimic the recalcitrance of real ones for pH values, concentration of dyes, additives and salts. In the culture condition with the lowest N content, the decolourisation was coupled with an important detoxification of two simulated effluents, underlining the important influence of the cultural medium composition not only on the degradation but also on the detoxification of industrial wastes. In the other cases, despite an extensive decolourisation, no detoxification was observed. The fungus was further tested against a real effluent, collected from a wastewater treatment plant before and after the tertiary treatment (ozonation) in order to compare the two technologies in terms of chemical and toxicological parameters. The fungal treatment, although less efficient than ozonation, caused a good decolourisation of the effluent, with colour values within the threshold limits of the Italian law; both the fungal and the ozone treatment caused a detoxification, but only towards one of the three organisms used for the ecotoxicological tests. These results underline the critical importance of the ecotoxicological analysis in assessing the applicability of a wastewater treatment.

Keywords: bioremediation, *Bjerkandera adusta*, ozonation, textile effluents, toxicity, white-rot fungi

Introduction

Increasing industrial development and urbanisation have resulted in generation of large quantities of toxic and persistent pollutants in watercourses that cause deleterious ecological effects and pose a serious threat to animals and humankind [1]. Considering both the volume and the composition of the effluents, the textile industry is rated as the most polluting among all industrial sectors. Textile effluents are one of the most difficult-to-treat wastewaters on account of the considerable amount of suspended solids and of the massive presence of dyes, salts, additives,

detergents and surfactants [2]. The physical-chemical technologies for wastewater treatment, including the advanced oxidation processes (i.e. application of ozone, hydrogen peroxide and ultraviolet), are expensive, not always effective, and often do not reduce the toxicity of the effluents [3].

In recent years, many studies on innovative biological approaches have investigated the possibility to use selected microorganisms to degrade dyes in wastewaters. Fungi, particularly white rot fungi, have long been recognised for their ability to degrade a wide range of recalcitrant compounds, such as synthetic dyes, through the use of relatively non-specific, extracellular oxidative enzymes [4, 5]. This extracellular enzymatic system, which in nature is involved in lignin degradation, consists mainly of oxidative enzymes like laccases (Lac), lignin peroxidases (LiP) and manganese peroxidases (MnP) that have been demonstrated effective against a wide range of industrial dyes [6].

However, fungal treatments have not yet found a real application, mainly due to the difficulty in selecting organisms able to grow and remain active in the very variable and harsh conditions of wastewaters [7]. In fact, fungi should be able to live with the scarce nutrient resources present in the effluents, to win the competition with the autochthonous microflora and to survive in presence of high concentrations of salts, dyes, detergents and heavy metals. Moreover, the requirement of low pH values (4-5), optimum enzyme activity, and long hydraulic retention times for complete decolourisation are other concerns in fungal treatments application [8].

Another important and often underestimated aspect is the reduction of toxicity at the end of the process that is a prerequisite to hypothesize a real application in the treatment of coloured wastewaters [7]. Actually, biodegradation does not imply that the resulting metabolites are less toxic than the parent molecules [9] and chemical procedures alone cannot provide sufficient information on the potential harmful effects of pollutants on the aquatic ecosystem. This can be done only by means of bioassays [10, 11, 12].

Different organisms have been standardized for ecotoxicity evaluation and the most suitable test organism should be selected taking into account the characteristics of the wastewaters (i.e. colour, turbidity) and of the receiving stream [13]. Since different species do not respond identically to the same pollutant and show varying sensitivity, a realistic representation of an ecosystem using a single organism is not achievable. The application of a battery of tests with organisms of different trophic level is always recommended for toxicity monitoring of wastewaters [13, 14].

In a previous study [7], the important influence of culture conditions on the efficiency of wastewater decolourisation and detoxification by a strain of *Bjerkandera adusta* (Willdenow) P. Karsten (MUT 2295) was highlighted. In this study a similar optimisation process was carried out on another strain of *B. adusta* (MUT 3060), which was effective in wastewater treatment by means of a different enzymatic pattern [data not shown]. In this way, it was also possible to investigate the eventual intraspecific variability of different strains of *B. adusta* in the decolourisation and detoxification of effluents. The wastewaters toxicity, before and after treatment, was analysed, in deeper detail than in the previous study [7], by means of an ecotoxicity tests battery using three organisms belonging to different trophic levels that showed a good or high sensitivity to the toxicity of textile wastewaters [15]. Finally, the fungus was tested against a real wastewater to assess its potential for bioremediation in terms of both decolourisation and detoxification.

Materials and Methods

Simulated and real textile effluents

Four simulated wastewaters, developed by the industrial partners of the EC FP6 Project SOPHIED (NMP2-CT-2004-505899), were designed to mime effluent recalcitrance for pH, concentration of dyes, additives and salts produced during wool (W1), cotton (W2, W3), and leather (W4) dyeing processes and were used under the permission of the SOPHIED Consortium. W1 (pH5) contained 100 ppm of each of the acid dyes AY49, AR266 and Abu62, and 2000 ppm of Na₂SO₄. W2 (pH 10) contained 1250 ppm of each of the reactive dyes RY145, RR195, Rbu222 and Rbk5, and 70000 ppm of Na₂SO₄; since reactive dyes enter textile effluents after thermal hydrolysis

of reactive groups occurring in dyeing-bath conditions, the dyes were previously hydrolyzed by a 4 h treatment at 95°C in a NaOH (10 g/l) solution. W3 (pH9) contained 1000 ppm of each of the direct dyes DrY106, DrR80, and DrBu71, and 5000 ppm of NaCl. W4 (pH5) contained 100 ppm of each of the acid dyes ABk194, Abk210 and AY194. The industrial dyes were kindly provided by Town End (Leeds, UK) plc. and all the other chemicals were purchased from Sigma Aldrich (St. Louis, MO USA). The simulated wastewaters were sterilised by tyndalization (three 1h cycles at 60 °C with 24 h interval between cycles at room temperature) before use.

The real wastewater was kindly provided by EURO D srl (BG, Italy), a company that treats about 4500 m³ per day of effluents mainly coming from cotton, polyester and polyamide dyeing processes, through a primary (chemico-physical), secondary (activated sludge) and tertiary process (ozonation). The effluent for fungal treatment experiments was sampled after the secondary treatment (WR pre O₃) and had the following features: pH 8.5, COD 145 mg/l, chlorides 780 mg/l, sulfates 200 mg/l. In addition, a sample of wastewater was collected after the tertiary treatment (WR post O₃) in order to compare the effects of the fungal treatment and the ozonation in relation to chemical and ecotoxicological parameters.

Organism

The strain tested in this study, *B. adusta* MUT 3060, is preserved at the *Mycotheca Universitatis Taurinensis* Collection (MUT, University of Turin, Department of Plant Biology) on Malt Extract Agar (MEA) at 4°C. The strain was selected in a previous study because of its efficient decolourisation activity against different dye classes and against W1-4, in the absence of any additional nutrient source [7].

Decolourisation experiments

In order to evaluate the effect of different culture conditions on the W1-4 decolourisation, the fungal treatment was carried out in presence and in absence of glucose and yeast extract as described below. The fungus was inoculated as five agar plugs (5 mm of diameter), taken from the edge of an actively growing colony on MEA, in 100 ml flasks containing 30 ml of a high nitrogen

content medium as previously described [7]. The flasks were incubated in agitated conditions (130 rpm) at 28°C. After 7 days, the culture broths were replaced with 30 ml of the four simulated wastewaters in presence and in absence of nutrients: 10 g l⁻¹ glucose and 3.18 g l⁻¹ of yeast extract (GHY); 10 g l⁻¹ glucose and 0.15 g l⁻¹ of yeast extract (GLY). Since the very restrictive conditions of W2 hindered the growth and activity of the fungus [7], a dilution of this wastewater to 1/3 and 1/5 was necessary. Heat-killed controls (obtained by autoclaving cultures before adding the dye) and abiotic controls (without fungal inoculum) were included. Each trial was performed in triplicate.

In the real wastewater experiment, the inoculum consisted in ten fungal plugs added, as described above, in 500 ml flasks containing 200 ml of GHY. After 7 days, the mycelium was recovered, homogenized using Ultra-Turrax (Ika, Germany), and inoculated (2.5 ml) in 200 ml flasks containing 100 ml of GHY. After 7 days, the culture broths were replaced with 100 ml of the real wastewater WR pre O₃ in presence of glucose and yeast extract in the proportion of GLY 1/10 (1 g l⁻¹ glucose and 0.015 g l⁻¹ of yeast extract).

Daily, 1 ml of wastewater from each sample was taken, centrifuged at 14,000 rpm for 5 min to remove disturbing mycelial fragments, and examined with a spectrophotometer (TECAN Infinite M200, Austria) to acquire the absorbance spectrum from 360 nm to 790 nm and to monitor the enzymatic activities as described in the following paragraphs. Since a linear relationship subsisted between the area of absorbance spectrum and dye concentration, the percentage of removed dye (decolourisation percentage, DP) was calculated (software Magellan V 6.5, TECAN, Austria) as the extent of decrease of the spectrum area of the treated effluent, with respect to that of the abiotic control. Significance of differences (P≤0.05) among DP values at the end of the experiment was calculated by the Mann-Whitney test (SYSTAT 10, SPSS inc. 2000).

Enzymes assays and native polyacrylamide gel electrophoresis

Laccase (Lac) activity was assayed at 25°C, following the oxidation at 420 nm of 2.2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), in 0.1 M sodium citrate buffer pH 3 [16].

Manganese-independent (MiP) and manganese-dependent (MnP) peroxidase activities were measured at 25°C, following the oxidation at 590 nm of 3-dimethylaminobenzoic acid/3-methyl-2-benzothiazolinone hydrazone hydrochloride (DMAB/MBTH), in 0.1 M succinate lactate buffer pH 4.5 [17]. For MnP, 25 µM MnSO₄ were added to the mixture. Lignin peroxidase (LiP) activity was assayed at 35°C, following the oxidation at 310 nm of veratryl alcohol, in 0.1 M sodium tartrate buffer pH 3.0 [18]. All the enzyme activities were expressed as International Units (IU), where 1 unit is defined as the amount of enzyme that oxidizes 1 µmole of substrate per minute.

The liquid cultures, concentrated by Amicon ultracentrifugal filters with 10000 Da cut off (Millipore, USA), were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli [19], with the omission of sodium dodecyl sulfate and β-mercaptoethanol. The gels were loaded with 0.03 IU per well and, after separation, were directly immersed in a solution containing 0.1 M sodium citrate buffer pH 3 and 2 mM ABTS for laccase activity and in the same solution, with the addition of 100 µM H₂O₂, for peroxidase activity.

Ecotoxicity tests and COD determination

Toxicity of the simulated wastewaters was evaluated, before and after the fungal treatment, by means of a battery of three tests. The phytotoxicity test on *Cucumis sativus* L. seeds was set up according to the method UNICHIM N. 1651 (2003). The germination index (GI%) was calculated according to the formula: $GI\% = (Gs \cdot Ls) / (Gc \cdot Lc) \cdot 100$, where Gs is the mean number of germinated seeds in the sample, Ls is the mean root length of the sample, Gc is the mean number of germinated seeds in the control (made of distilled water), Lc is the mean root length of the control.

The inhibition of cellular growth of the green unicellular alga *Pseudokirchneriella subcapitata* (Korshilov) Hindak was performed according to the method UNI EN ISO 8692:2005. The results were expressed as percentage of inhibition of the algal growth (I%) comparing the treated samples with a control made up by nutrient solution only.

The test on *Vibrio fischeri* was set up according to the method UNI EN ISO 11348-3:2001. The luminescence of the bacteria in the treated samples, measured by Microtox (Microtox Model

500; Microbics Corp., USA) after 15 min exposure at 15°C, was compared to that of a non-toxic control made of the Microtox diluent solution only. Correction for colour, which might have an impact on the luminescent measurements, was performed on all samples according to the MicrotoxOmni manual. Toxicity was expressed as the effective concentration that inhibits the bacterial growth by 50% (EC₅₀), and as its inverse (Toxicological Units, TU₅₀). Thanks to the reduced amount of material necessary for this test (about 5 ml of composite sample from 3 repetitions), it was possible to conduct this analysis not only at the beginning and at the end of the treatment, but also at an intermediate time (7 days).

Determination of the chemical oxygen demand (COD) with the dichromate method was performed before and after treatment (IV cycle) of WR pre O₃ effluent using HACH (COD High range vials) apparatus (HACH LANGE, S.r.l. Milan, Italy) according to the manufacture's instructions. A calibration curve was obtained using HACH COD standard solution (800 mg l⁻¹ of O₂). Appropriate dilutions of each sample were assayed.

All data were expressed as average (± conf. 95%). The *V. fischeri*, *P. subcapitata* and COD analysis of the real effluent were conducted by ARPA Piemonte that, in accordance with its internal procedures, does not provide confidence limits when the sample is within the threshold limit of the Italian law (Legislative Decree 152/2006).

Results and discussion

B. adusta MUT 3060 has proved to be a strong and versatile organism, able to grow in very variable and harsh conditions (pH from 5 to 10, salts up to 70 g l⁻¹ and 13 different dyes up to a concentration of 5000 ppm). The fungus resulted very effective in the decolourisation of different wastewaters (up to 96% of DP, Table 1). As already described by other authors [20, 21], a two-steps degrading process was observed during W1-4 decolourisation: an initial rapid decolourisation step, due to the adsorption of the dyes on the mycelium that resulted visibly coloured, was followed by an effective degradation step, highlighted by the complete knock down of the absorption spectra (Figure 1) and by the resulting colourless biomasses. This phenomenon was confirmed by killed

samples analysis: within the first hours, they caused a rapid reduction of the spectra as well (about 30% of DP); however, unlike living biomasses, they remained strongly coloured until the end of the experiment, in the absence of any degradation activity (Figure 2).

Different culture conditions resulted essential for the process optimisation, as already observed on another strain of *B. adusta* against the same simulated wastewaters [7] and on *Pleurotus ostreatus* against W1 [22]. The addition of GLY strongly improved the decolourisation efficiency (DP values always higher than 90%, Table 1). The influence of the medium composition was particularly evident towards W2 1/5 (DP from 36 to 91%) and W3 (DP from 50 to 90%), indicating that the higher the wastewater recalcitrance, the more stringent the conditions for the growth and activity of the fungus become.

As regard to the enzymes produced during the decolourisation process, MnP and MiP activities were mainly detected, as generally reported in literature for this species [7, 23, 24], while Lac and LiP activities were almost absent or negligible (<5 U/l) (Table 1). No enzyme activity was detected in killed and abiotic controls. A good correlation was often observed between the main enzymatic activity, MnP, and DP as can be seen for example in the case of W4 GLY (Figure 3). The positive correlation between ligninolytic enzymes and decolourisation, as already observed by other authors [5], shows that these enzymes play a key role in the biodegradation of synthetic dyes and textile effluents though little is known about their combined role and interactions [25]. This is mainly due to the high complexity of the biodegradation mechanisms involved, where in addition to the ligninolytic enzymes, other biochemical systems may influence the rate of the bioremediation process, namely cytochrome P450 mono-oxygenase system, hydroxyl radicals and the level of H₂O₂ produced by the fungi [26]. However, the measure of high levels of activity of a specific enzyme, together with the removal of a pollutant, could be considered indicative of a relation between the detected enzyme and the degradation [26].

The MiP activity could indicate the involvement of different enzymes, including versatile peroxidase (VP), a new family of ligninolytic peroxidases with intermediate features between MnP

and LiP [27], or Dye-decolorizing peroxidase (DyP) [28]. Recently, DyP and VP were purified and characterized in *B. adusta* [29, 30]. However, the precise identification of these peroxidases and a clear distinction in classes are still complex because, as recently reviewed by Hofrichter [31], they belong to a very heterogeneous family, including enzymes with similar, partially overlapping, biochemical features.

Interestingly, as already described in other studies on this species [7, 23, 24], we also observed that the peroxidase production is greatly influenced by the environmental conditions (*i.e.* N content, wastewater composition, pH, etc.) [4] and that a higher enzyme activity does not always lead to a more efficient decolourisation, as in the case of W3 GLY and GHY (Table 1). This last aspect could be partly explained considering the pH values measured during the experiments and the important role of pH for the functioning of the enzymes, as already observed by other authors [32]. The best DPs were often achieved when the fungal metabolism caused a variation of pH to values around 5-6 (*optimum* for peroxidases). Another possible explanation could be the production, in different culture conditions, of enzymes with different affinity for dyes. In fact, this strain produced a different enzymatic pattern according to the wastewater (composition and dilution) and the cultural condition, as shown, for example, by the native PAGE on MiP activities produced on GLY, in presence and in absence of the different wastewaters (Figure 4). Further studies are necessary in order to identify more precisely these enzymes and to clarify their role in the decolourisation process.

Since decolourisation does not imply that the molecules resulting from the degradation process are less toxic than the parent ones [9], after the fungal treatment we monitored the toxicity of the wastewaters using two organisms (*C. sativus* and *P. subcapitata*). In presence of the lowest N content (GLY), the most efficient decolourisation was also coupled with an important detoxification of two effluents: W1 towards both organisms (*P. subcapitata* also in the other culture conditions), W2 1/3 towards *P. subcapitata* and W2 1/5 towards *C. sativus* (Table 2). In the case of W1 GLY, it is important to note that the fungal treatment led to a detoxification within the threshold limit values

of the Italian law ($GI\% > 50$ and $I\% < 50$ - Legislative Decree 152/2006). This result underlines the important influence of the cultural medium composition not only on the degradation but also on the detoxification of industrial wastewaters. On the contrary, despite a great decolourisation of W3 and W4, no detoxification was obtained; indeed, an increased toxicity was often observed (Table 2).

Concerning the test on *V. fischeri*, the toxicity measurement at mid-treatment provided a significant additional information. For all the tested samples, a marked increase of toxicity was noted during the first week. Then, in most cases the toxicity did not change or even decreased during the second week (Figure 3). This trend could be explained assuming that at the beginning *B. adusta* MUT 3060 breaks the chromophore structure causing the loss of colour; afterwards it goes on degrading the secondary products which could be more toxic than the dye itself. This hypothesis is supported by the enzymatic activity that remained at high levels even after the complete decolourisation of the wastewater, as can be seen for example in the case of W4 GLY (Figure 3).

The colour of the real wastewater WR pre O3 was reduced by 63% and 40% through ozonation (WR post O3) and fungal treatment, respectively (Table 2, Figure 5). Even if with a lower yield than ozone, the treatment with *B. adusta* MUT 3060 determined a good decolourisation, within the Italian threshold limit (Legislative Decree 152/2006, colour not visible after 1/20 dilution). From an applicative point of view, it is interesting to consider the duration of the fungal treatment, which resulted very rapid for a biological process (24 hours). In order to exploit the potential of decolourisation of both processes, a combined ozonation/fungal treatment may be performed, as also suggested by Vanhulle and collaborators (2008).

The COD value after fungal treatment resulted slightly above the threshold limit value for wastewater discharge (160 mg/l COD, Legislative Decree 152/2006), probably in consequence of the culture medium addition. The ecotoxicity tests showed different results according to the organism tested (Table 2). Only the fungal treatment led to a complete detoxification towards *C. sativus* ($GI\% = 99.8$), while an increased toxicity against *V. fischeri* and *P. subcapitata* was observed after both treatments; however, the values were still within the legal threshold limit,

except for *P. subcapitata* that resulted completely inhibited (I% = 100) after fungal treatment. It should be noted that, in addition to the toxicity of metabolites produced by the fungus, the strong inhibition of algal growth may depend on the lower pH values observed after fungal treatment; actually, *P. subcapitata* requires pH values around 8-8.5 for optimal growth. In the light of this, further analysis should be carried out to evaluate the contribution of pH to algal toxicity and, eventually, to identify toxic metabolites.

The results of the present study underline, in compliance with increasingly stringent environmental regulations, the importance of extensive ecotoxicological analysis to assess the applicability of a wastewater treatment process. This is true also when different strains of the same fungal species are used, as can be observed when we compare the results of this study with those obtained by the other strain of *B. adusta* [7]: the two strains showed a similar decolourisation efficiency, but a different capacity of detoxification towards the same simulated effluents probably in consequence of the different enzymatic pattern.

Our data clearly indicate that not only in the biological process through *B. adusta*, but also in the ozonation process, an effective decolourisation is often not coupled with a reduction of toxicity. In this context, it is important the use of organisms belonging to different trophic levels in order to achieve a more complete toxicological analysis. However, the difficulty of interpreting the often conflicting results of different tests, stresses the need to identify in the near future the most appropriate assays for different types of wastewaters; as demonstrated by recent studies [15], the seaweed *P. subcapitata* seems to be the most sensitive and therefore most suitable organism for textile wastewaters.

In conclusion, this work has shown that it is relatively easy to optimize the fungal decolourisation process, i.e. by means of media engineering, but not the detoxification. Further investigations are needed to understand in more detail the fungal metabolism involved in the degradation and to optimize the process on both sides: decolourisation and detoxification. One of the strategies to exploit the fungal oxidative potential could be to consider a longer treatment, in

order to obtain a higher rate of detoxification, or to intervene with the fungal treatment at an earlier stage, for example before the secondary treatment performed with conventional activated sludge. When we turn to fungi in natural environments, we find that they are particularly important in the breakdown and recycling of the most complex and recalcitrant polymers, such as lignocellulose. Such a strong oxidative capacity could be exploited at the early stages of the wastewater treatment, when fungi could detoxify the effluent degrading molecules toxic and recalcitrant for bacteria, and at the same time, reducing a significant part of the COD. Subsequent activated sludge stage could be operated to reduce the remaining COD and toxicity.

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Figure captions

FIGURE 1. Absorbance spectra of 4 simulated wastewaters (W1-4) in presence of GLY before and after treatment with *B. adusta* MUT 3060.

FIGURE 2. Aspect of living (a) and killed control (b) biomasses of *B. adusta* MUT 3060 at the end of the treatment of W1 GLY.

FIGURE 3. Decolourisation percentage (DP), main enzymatic activity, MnP (U/l), and *Vibrio fischeri* toxicity (TU₅₀) during treatment of W4 GLY by *B. adusta* MUT 3060.

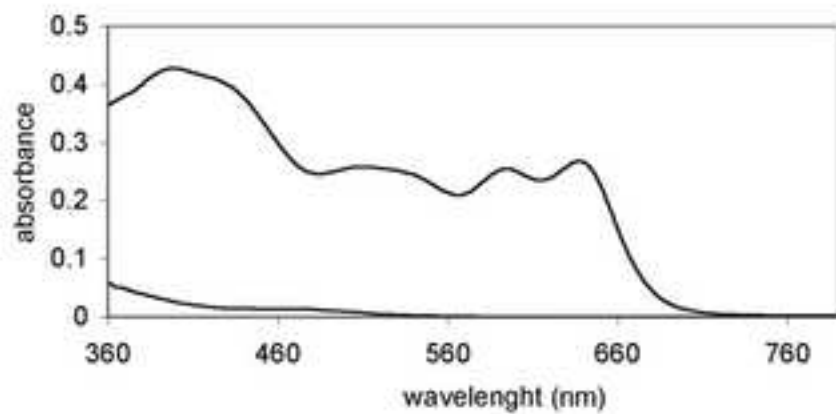
FIGURE 4. Native PAGE showing the peroxidase pattern in the culture liquid of *B. adusta* MUT 3060 in the absence (GLY) and in the presence of 4 simulated wastewaters (W1-4 GLY).

FIGURE 5. Absorbance spectrum of the real wastewater before (WR Pre O₃) and after treatment with ozone (WR Post O₃) and with *B. adusta* MUT 3060.

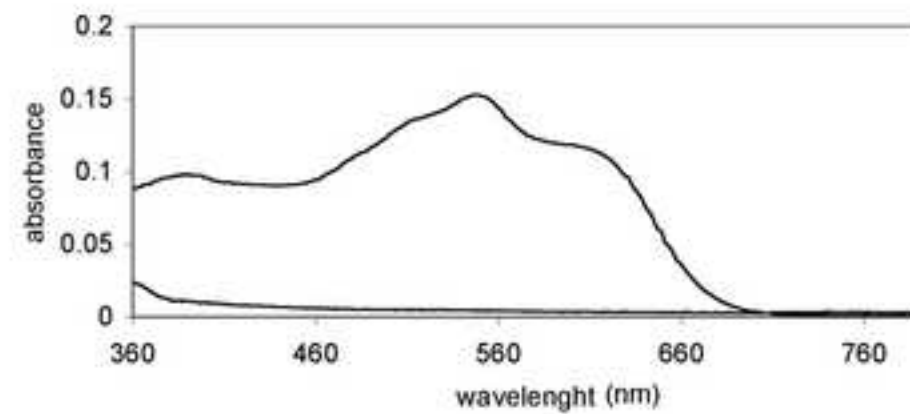
Figure

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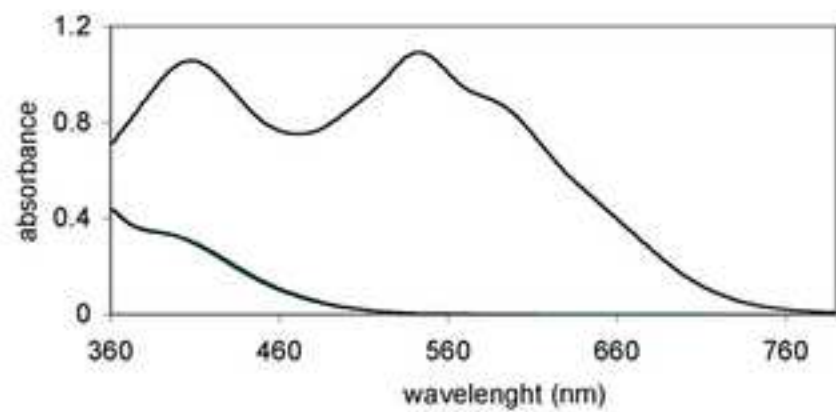
W1 GLY



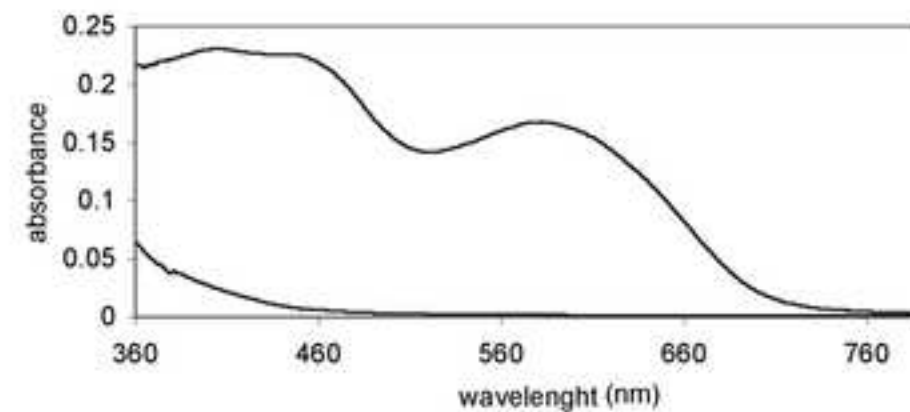
W2 1/5 GLY



W3 GLY



W4 GLY



Figure

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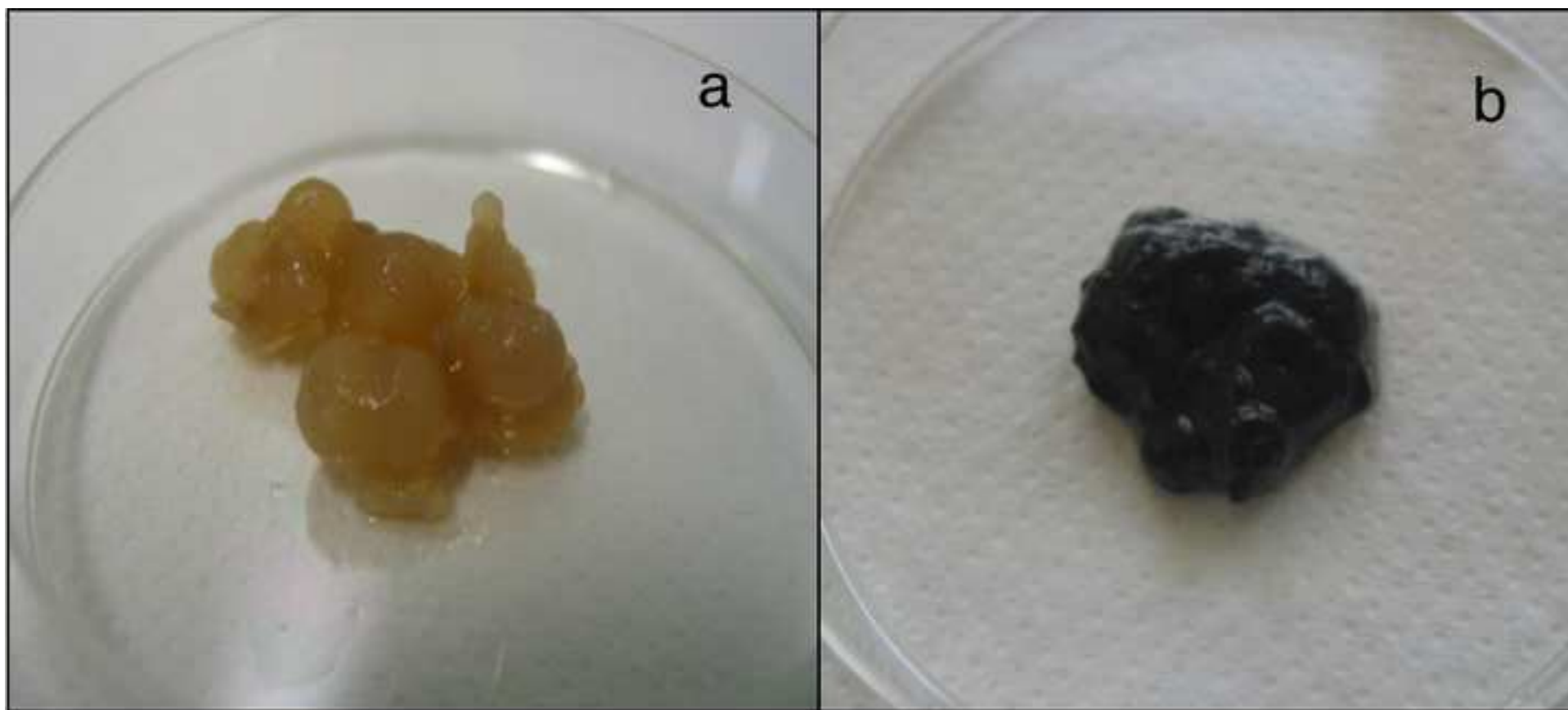
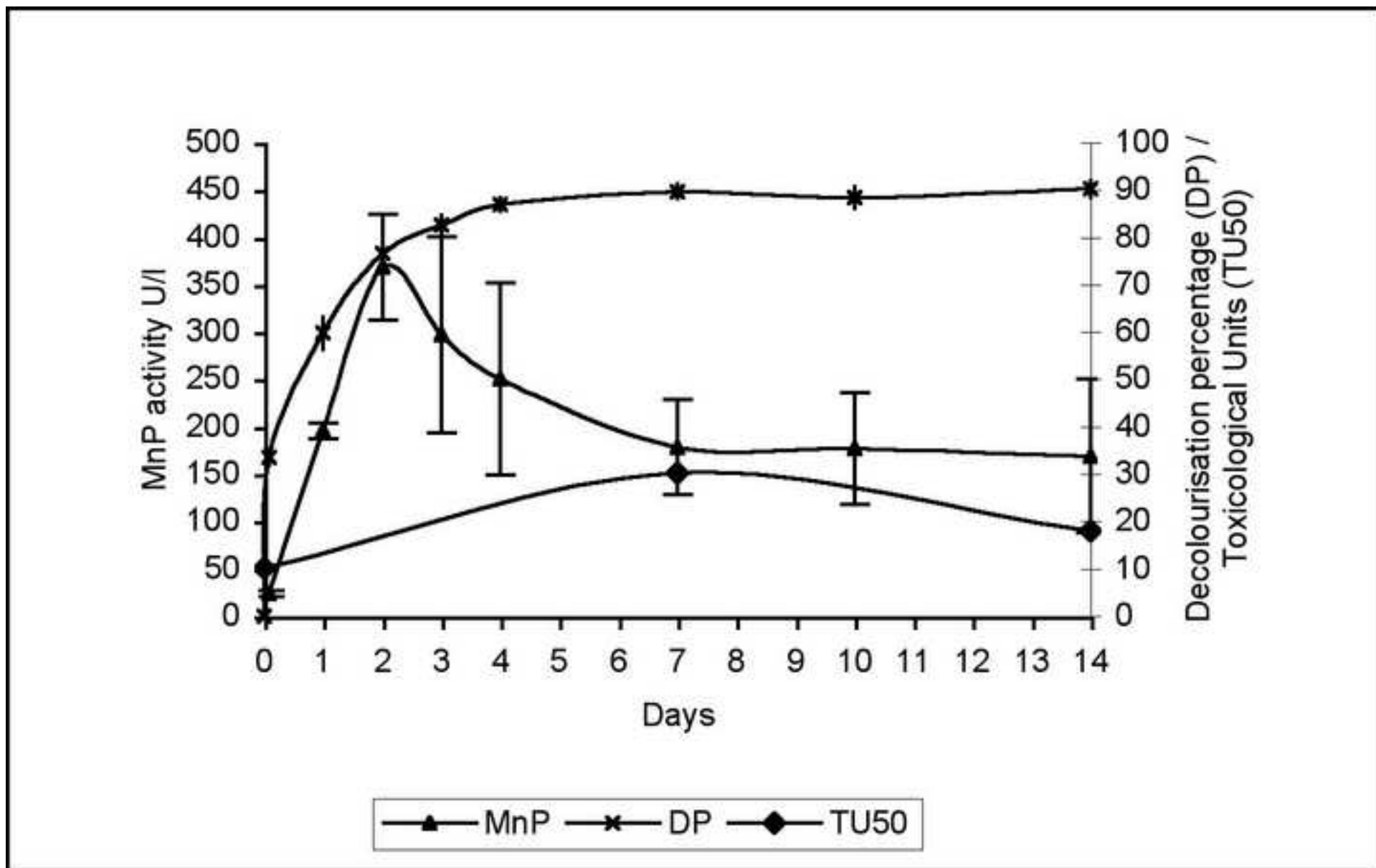


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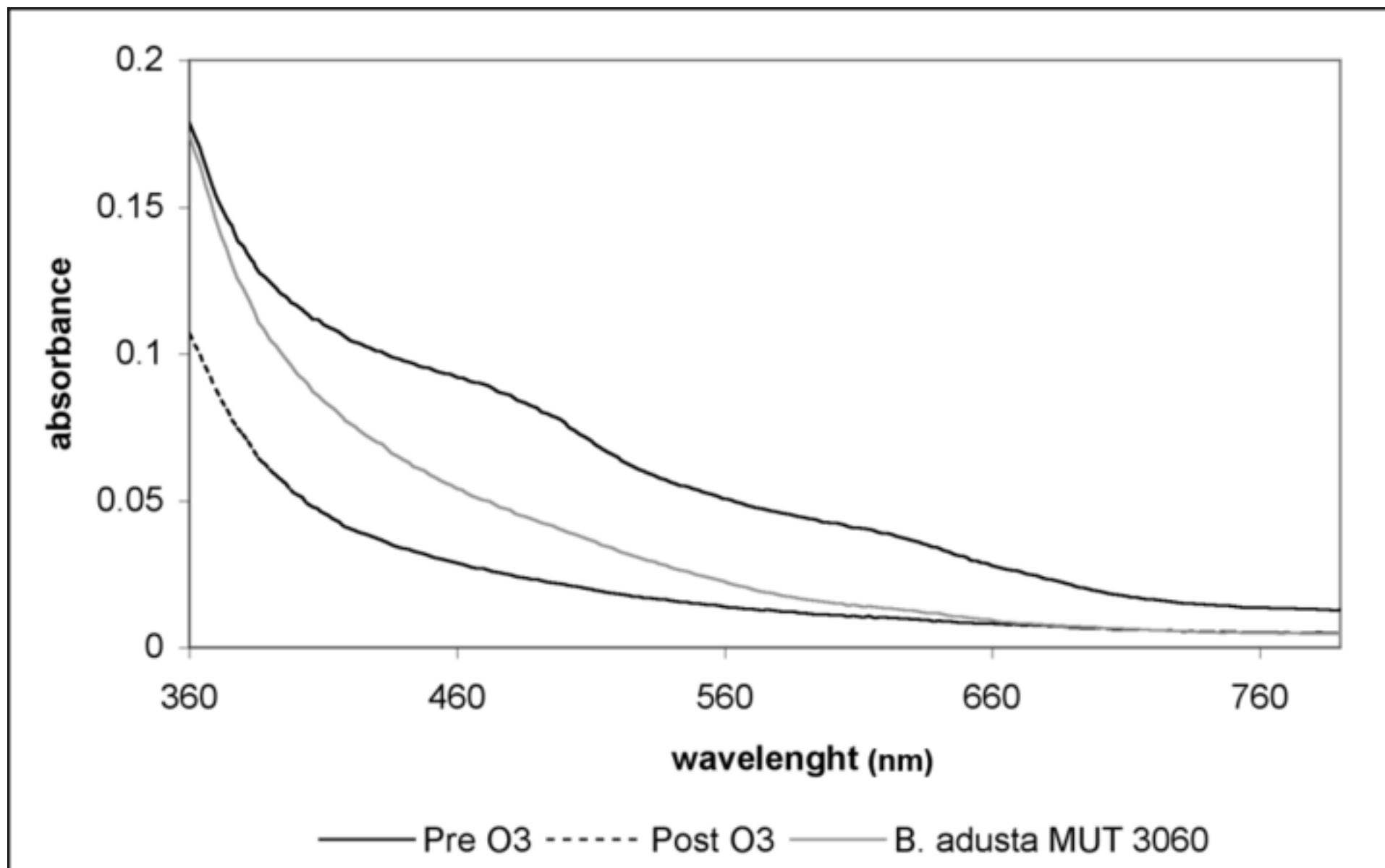


TABLE 1. Maximum values (\pm SD) of DP and peroxidase (MiP and MnP) activities (in brackets the day of measure) and pH values during treatment of the simulated wastewaters (W1-4) by *Bjerkandera adusta* MUT 3060, in the presence and in the absence of GLY/GHY.

	DP	MnP	MiP	pH
W1	88.0 \pm 1.4 (7)	291.1 \pm 6.2 (7)	45.2 \pm 1.6 (7)	5.7
W1 GLY	96.5 \pm 0.5 (3)	341.9 \pm 66.8 (7)	54.7 \pm 17.3 (7)	5.8
W1 GHY	67.5 \pm 1.9 (3)	218.8 \pm 45.9 (4)	74.6 \pm 12.9 (4)	7.5
W2 1/3	29.2 \pm 2.3 (1)	41.9 \pm 3.8 (3)	16.2 \pm 0.4 (7)	6.8
W2 1/3 GLY	55.8 \pm 4.1 (15)	239.3 \pm 34.5 (14)	33.6 \pm 9.3 (14)	6.3
W2 1/3 GHY	61.0 \pm 3.3 (10)	736.4 \pm 71.7 (10)	80.4 \pm 4.8 (7)	6.2
W2 1/5	36.4 \pm 6.0 (2)	66.5 \pm 19.6 (7)	9.7 \pm 6.2 (7)	5.9
W2 1/5 GLY	91.0 \pm 6.5 (10)	255.4 \pm 43.1 (7)	70.5 \pm 4.0 (7)	5.4
W2 1/5 GHY	61.9 \pm 6.3 (10)	220.3 \pm 42.1 (7)	36.3 \pm 11.0 (7)	6.9
W3	50.2 \pm 7.0 (1)	195.1 \pm 34.3 (2)	34.2 \pm 10.0(0.1)	7.4
W3 GLY	89.7 \pm 1.5 (7)	368.3 \pm 55.9 (2)	36.9 \pm 13.5 (2)	6.5
W3 GHY	75.0 \pm 0.3 (4)	558.4 \pm 20.9 (2)	97.8 \pm 4.3 (2)	8.6
W4	82.7 \pm 7.8 (1)	281.0 \pm 57.2 (10)	32.1 \pm 8.4 (4)	6.6
W4 GLY	91.4 \pm 2.5 (4)	196.7 \pm 25.6 (1)	20.4 \pm 7.5 (1)	7.3
W4 GHY	74.6 \pm 2.8 (2)	281.4 \pm 36.0 (2)	79.6 \pm 14.0 (2)	8.2

TABLE 2. Values (\pm conf. 95%) of *Cucumis sativus* germination index (GI%, method UNICHIM N. 1651 2003), *Pseudokirchneriella subcapitata* growth inhibition (I%, method UNI EN ISO 8692:2005) and *Vibrio fischeri* EC₅₀ (method UNI EN ISO 11348-3:2001) of the simulated wastewaters (W1-4) before and after treatment by *Bjerkandera adusta* MUT 3060, in the presence and in the absence of GLY/GHY. Different letters indicate significant differences ($p \leq 0.05$) of values before and after fungal treatment.

	<i>C. sativus</i>		<i>P. subcapitata</i>		<i>V. fischeri</i>		
	GI% before fungal treatmentt	GI% after fungal treatment	I% before fungal treatment	I% after fungal treatment	EC ₅₀ before fungal treatment	EC ₅₀ after 7 d fungal treatment	EC ₅₀ after 14 d fungal treatment
W1	56.7 \pm 5.0a	64.5 \pm 8.8a	68.9 \pm 5.2a	59.6 \pm 3.0b	38.8 \pm 2.0a	5.4 \pm 0.1b	5.1 \pm 0.1 b
W1 GLY	46.1 \pm 4.8a	72.9 \pm 6.6b	89.5 \pm 10.3a	0.9 \pm 0.2b	25.2 \pm 0.1a	11.2 \pm 0.2b	6.4 \pm 0.3b
W1 GHY	52.1 \pm 3.1a	27.5 \pm 3.3b	48.5 \pm 14.5a	24.7 \pm 9.8a	52.5 \pm 1.5a	5.9 \pm 0.03b	13.7 \pm 0.6b
W2 1/3	5.3 \pm 1.7a	5.7 \pm 1.8a	89.5 \pm 8.5a	90.9 \pm 9.3a	18.3 \pm 0.3a	5.3 \pm 0.1b	5.0 \pm 0.2b
W2 1/3 GLY	3.4 \pm 1.9a	4.3 \pm 2.1a	76.9 \pm 3.2a	68.7 \pm 2.1b	5.6 \pm 0.2a	4.9 \pm 0.6ab	3.1 \pm 0.03b
W2 1/3 GHY	4.4 \pm 1.8a	3.0 \pm 0.7a	36.9 \pm 1.8a	82.4 \pm 6.7b	17.6 \pm 0.2a	3.2 \pm 0.1b	2.7 \pm 0.04b
W2 1/5	32.4 \pm 1.8a	28.1 \pm 1.4b	45.4 \pm 6.8a	75.2 \pm 9.4b	14 \pm 0.4a	2.7 \pm 0.02b	*
W2 1/5 GLY	24.1 \pm 2.1a	28.5 \pm 2.6b	67.4 \pm 7.1a	75.5 \pm 8.3a	7.7 \pm 0.1a	9.7 \pm 0.1b	*
W2 1/5 GHY	29.2 \pm 2.2a	21.1 \pm 1.4b	62.5 \pm 5.0a	58.4 \pm 7.2a	22.3 \pm 0.2	*	*
W3	75.5 \pm 10.4a	47.3 \pm 7.0b	35.8 \pm 3.3a	92.8 \pm 8.5b	*	3.2 \pm 0.2	4.7 \pm 0.3
W3 GLY	64.7 \pm 9.4a	66.0 \pm 9.5a	-19.3 \pm 4.4a	7.8 b \pm 2.1	9.8 \pm 3.5a	3.3 \pm 0.3b	5.5 \pm 0.8b
W3 GHY	58.0 \pm 8.2a	42.5 \pm 5.3b	-1.6 a \pm 0.3	43.4 \pm 5.9b	5.1 \pm 1.1a	2.2 \pm 0.2a	4.6 \pm 1.2a
W4	122.1 \pm 4.5a	49.0 \pm 3.2b	29.1 \pm 3.2a	73.3 \pm 5.6b	18.4 \pm 0.3a	5.0 \pm 0.04b	14.1 \pm 0.01b
W4 GLY	92.9 \pm 9.0a	73.6 \pm 7.4a	78.5 \pm 4.3a	86.6 \pm 7.4a	16.4 \pm 0.1a	2.2 \pm 0.03b	12.3 \pm 0.2c
W4 GHY	99.0 \pm 6.1a	43.3 \pm 2.8 b	48.8 \pm 3.1a	91.9 \pm 8.4b	2.2 \pm 0.1a	1.9 \pm 0.1b	15.2 \pm 0.3b

* insufficient amount of sample to conduct the test.

TABLE 3. Decolourisation percentage (DP \pm SD, in brackets the time of measure), COD, pH and ecotoxicological analysis (\pm conf. 95%) of the real wastewater before (WR Pre O₃) and after treatment with ozone (WR Post O₃) and with *B. adusta* MUT 3060.

	WR Pre O₃	WR Post O₃	<i>B. adusta</i> MUT 3060
PD	-	63.0 \pm 3.2 (15')	40.8 \pm 6.8 (24 h)
COD (mg/l)	145.0 *	133.0 *	180.0 \pm 26
pH	8.1 *	7.9 *	7.3 *
<i>V. fischeri</i> I%	12.7 *	36.2 *	40.6 *
<i>P. subcapitata</i> I%	19.0 *	34.3 *	100.0 – 26.7
<i>C. sativus</i> GI%	38.0 \pm 3.9	42.1 \pm 8.0	99.8 \pm 18.2*

* values within the threshold limit of the Italian law (Legislative Decree 152/2006).