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Integrated fungal biomass and activated sludge treatment for textile wastewaters bioremediation

Antonella Anastasi, Federica Spina, Alice Romagnolo, Valeria Tigini, Valeria Prigione, Giovanna Cristina Varese

Abstract

A combined biological process was investigated for effective textile wastewater treatment. The process consisted of a first step performed by selected fungal biomasses, mainly devoted to the effluent decolourisation, and of a subsequent stage by means of activated sludge, in order to reduce the remaining COD and toxicity. In particular, the treatment with *Trametes pubescens* MUT 2400, selected over nine strains, achieved very good results in respect to all parameters. The final scale-up phase in a moving bed bioreactor with the supported biomass of the fungus allowed to verify the effectiveness of the treatment with high volumes. Despite promising results, further steps must be taken in order to optimize the use of these biomasses for a full exploitation of their oxidative potential in textile wastewater treatment.

1. Introduction

The supply of clean freshwater is critical to the future of man and indeed to that of the biosphere itself (Johnston, 2003). In recent years, there has been an intensive research in industrial wastewaters treatments, in order to develop more effective technologies and to reduce the release of toxic and polluting substances in watercourses.

Considering both the volume and the composition of the effluents, the textile industry is rated as one of the most polluting among all industrial sectors. Textile effluents are one of the most difficult-to-treat on account of the considerable amount of suspended solids and of the massive presence of dyes, salts, additives, detergents, and surfactants (Prigione et al., 2008). Traditional technologies include various physical and chemical processes (primary treatments) coupled with a secondary biological treatment performed by activated sludge. These methods are often ineffective for wastewater decolourisation and a tertiary treatment is necessary (i.e. ozonation, photochemical processes). These additional methods, however, are very expensive and not always solve the problem of toxicity (Vanhulle et al., 2008).

In recent years, many attempts have been made to obtain a higher efficiency of treatment by modifying conventional treatment process with additional physical, chemical and/or biological processes (Park et al., 2011); studies on innovative biological approaches have investigated the possibility to use selected microorganisms in order to degrade dyes in wastewaters.

Fungi, particularly white rot fungi, have long been recognized 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 (Kaushik and Malik, 2009 and Singh and Arora, 2011). This 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 recognized as effective against a wide range of industrial dyes (Husain, 2006). Recently, the low efficiency of dye removal by mixed bacterial communities and the high rates of dye decolourisation by white-rot fungi suggest a combination of both processes as an option of treatment of textile wastewater containing dyes and high concentrations of organic compounds (Novotny et al., 2011).

Once a promising fungal organism is selected, a critical point for its exploitation in wastewater treatment at an industrial scale is the development of suitable technologies, able to maintain the metabolic activity of the organism in very harsh conditions. The use of several bioreactors, mainly fixed bed reactors, has been investigated for dye decolourisation by white rot fungi (Blanquez et al., 2008 and Novotny et al., 2011). Only recently, the moving bed system has been investigated (Park et al., 2011). This technology is based on specially designed carriers colonized by the fungus that are suspended and in continuous movement within a tank or reactor. It could be particularly advantageous because it can handle high loading conditions without any problem of clogging and on a relatively small footprint.

The aim of the present paper was to investigate the possibility to integrate the conventional biological treatment, normally performed by activated sludge, with a biodegradation phase by means of fungal biomasses, in order to obtain a more effective treatment of the textile effluent. The first step was aimed to the selection, over nine fungi, of the most effective ones in terms of reduction of colour, COD and toxicity of a wastewater sample collected from the neutralization tank of a dyeing plant. The best strains were then tested, both as free and supported biomass, in a sequential treatment in flasks in which, after the first step of fungal degradation, primarily addressed to decolourisation, the wastewater was subjected to a second step by means of activated sludge, mainly aimed at the reduction of residual COD and toxicity. Finally, the best strain was packed in a moving-bed bioreactor (MBBR), in order to treat larger volumes of wastewater.

2. Methods

2.1. Organisms

Nine white-rot fungi were selected from previous experiments (Anastasi et al., 2010 and Casieri et al., 2010) for their degradation capability towards model and industrial dyes: *Bjerkandera adusta* (Willd.) P. Karst. MUT 3060, *B. adusta* MUT 2295, *Corioloopsis gallica* (Fr.) Ryvarden MUT 3379, *Ganoderma adspersum* (Schulzer) Donk MUT 3427, *Porostereum spadiceum* (Pers.) Hjortstam & Ryvarden MUT 1585, *Steccherinum ochraceum* (Pers.) Gray MUT 3435, *Trametes hirsuta* (Wulfen) Lloyd MUT 3438, *T. pubescens* (Schumach.) Pilát MUT 2400, *T. versicolor* (L.) Lloyd MUT 3403. The strains belong to species ascribable to different families (Ganodermataceae, Meruliaceae, Phanerochaetaceae, Polyporaceae); they are lignicolous saprotrophs isolated both from carpophores and compost. The fungi are preserved at the *Mycotheca Universitatis Taurinensis* Collection (MUT, University of Turin, Department of Plant Biology).

2.2. Wastewaters

The wastewaters (W1, W2 and W3) were sampled in March, June and December 2011, respectively, from the neutralization tank of a textile dyeing plant that treats about 3000 m³ per day of effluents mainly coming from cotton dyeing industries, through a primary (chemico-physical), secondary (activated sludge) and tertiary process (ozonation). The effluents parameters were the following: pH 11.9 and 370 mg/l of COD (W1); pH 10.4 and 400 mg/l of COD (W2); pH 11 and 278 mg/l of COD (W3). In all cases, due to the very restrictive conditions for fungal growth, pH was lowered to 7 using HCl and 100 mg/l of glucose were added, causing an increase of COD estimated at 115 mg/l. Together with the effluents, from the secondary treatment of the same plant, samples of activated sludge (AS) were also sent after sedimentation.

2.3. W1 treatment by nine fungal strains

Fungi were inoculated as 20 agar plugs (5 mm of diameter), taken from the edge of an actively growing colony on MEA, in 500 ml flasks containing 200 ml of a high nitrogen content medium as previously described (Anastasi et al., 2010). The flasks were incubated in agitated conditions (130 rpm) at 28 °C. After 7 days, the culture broths were replaced with 100 ml of W1. In addition, in order to compare the effectiveness of the fungal treatment with the secondary treatment used in the plant, a line (AS) containing the effluent (100 ml) and 20 ml of sedimented AS was set up. An abiotic control (without fungal inoculum) was included and each trial was performed in triplicate. Due to the high number of strains, a composite sample from the three replicates was produced in order to perform the analysis of decolourisation, COD, enzymes activity and detoxification as described in the following paragraphs.

2.4. W2 treatment by free and supported biomasses of three strains

The wastewater treatment was performed in two steps, of 48 h each. During step 1 (fungal treatment) the best three strains selected from the previous experiment (*B. adusta* MUT 2295, *P. spadiceum* MUT 1585, *T. pubescens* MUT 2400) were inoculated as described above in 500 ml flasks containing 200 ml of the high nitrogen content medium. After 7 days, the mycelium was recovered, homogenized using Ultra-Turrax (Ika, Germany), and inoculated (5 ml) in 500 ml flasks containing: 200 ml of the same medium (free biomass, F) or 200 ml of the same medium and 12 polyurethane foam carriers constituted by cubes of about 2 cm side (supported biomass, S, Fig. 1), corresponding to a loading factor (volume of carriers/volume of the effluent) of around 50%. The flasks were incubated in agitated conditions (130 rpm for F and 80 rpm for S) at 28 °C. After 7 days, the culture broths were replaced with 150 ml of W2. Abiotic controls and AS line as described above were included. Per each culture line, four flasks were set up, and immediately after the preparation, one flask was sacrificed for starting analysis (t₀) of the different parameters. After 48 h, at the end of step 1, the biomass of each sample was filtered on paper filters (Wathman type 1) and dried at 65 °C for dry weight calculation; after collection of samples for chemical and toxicological analysis, the remaining effluent was used for the second step treatment.

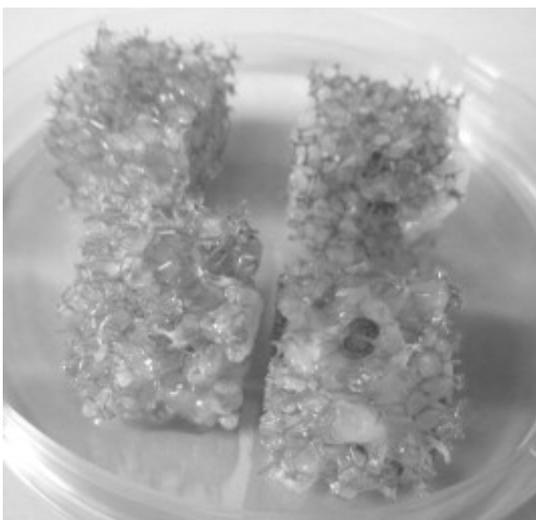


Fig. 1.

Polyurethane foam carriers colonized by *Trametes pubescens* MUT 2400.

During step 2 (AS treatment), 20 ml of sedimented AS were inoculated in the effluent (100 ml) coming from the six culture lines, after the fungal treatment. The experiment was performed in

triplicates and flasks were incubated as described above for 48 h. At the end of the experiment, the biomass was filtered and dried for dry weight calculation.

2.5. W3 treatment by fungal MBBR

The best strain selected from the previous experiment (*T. pubescens* MUT 2400) was grown on supports as previously described. After 7 days, 120 colonized supports were put in a 5 l bioreactor (Biostat B plus – 5L MO, Sartorius) containing 2 l of W3. No mechanical agitation was provided; stirring and homogeneity of the effluent were ensured by aeration (2 l/min) from the bottom of the tank. The bioreactor was kept at room temperature (about 25 °C) for 48 h. Periodically, 1 ml of wastewater was withdrawn from the system for decolourisation and enzyme activities monitoring.

The culture volume was also scaled-up in flasks, with the same loading factor: 10 supports in 167 ml of effluent (flask A); 30 supports in 500 ml of effluent (flask B). The flasks were incubated at 25 °C in an orbital shaker at 80 rpm. Abiotic controls and AS line as described above were included. After the fungal treatment (phase 1), the liquid was separated and inoculated in presence of AS (phase 2), as in the previous experiment.

2.6. Decolourisation analysis

Daily, 1 ml of wastewater from each culture line was taken, centrifuged at 14,000 rpm for 5 min in order to remove mycelial fragments, and examined with a spectrophotometer (TECAN Infinite M200, Austria) to acquire the absorbance spectrum from 360 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.

2.7. Enzyme activity assays

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 (Niku-Paavola et al., 1988). MnP and manganese-independent peroxidase (MiP) 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. For MnP, 25 µM MnSO₄ were added to the mixture (Vyas et al., 1994). All the enzyme activities were measured in 96-well, flat-bottom microtiter plates, using a spectrophotometer (TECAN Infinite M200, Austria) and were expressed as International Units (U), where one unit is defined as the amount of enzyme that oxidizes 1 µmol of substrate per minute.

2.8. Ecotoxicity tests and COD determination

Toxicity of W2 was evaluated, before and after fungal and AS treatment (step 1 and 2), by means of the test on the green unicellular alga *Pseudokirchneriella subcapitata* (Korshilov) Hindak (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. On account of the wide range of toxicity of the wastewaters, different dilutions were considered.

Due to the lower availability of liquid, toxicity of W3 was evaluated by means of a test that requires lower sample volumes. The phytotoxicity test on *Lepidium sativum* seeds was set up, before and

after fungal and AS treatment (step 1 and 2), 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 (consisting of distilled water), Lc is the mean root length of the control.

3. Results and discussion

The screening in liquid condition allowed to verify the potential application of the nine fungi for wastewater treatments. During W1 treatment, the nine fungal strains showed in general a good adaptation to the wastewater. A very important aspect to consider is the fact that generally the metabolic capacity of fungi decreases in the absence of sterile conditions, as demonstrated by Borchert and Libra (2001). In our case, the fungal strains remained active even in the presence of the microflora associated with the real dyeing wastewater.

As can be seen in Table 1, the decolourisation yields were already very high within the first 24 h, in particular for *T. pubescens* (76% DP), *T. versicolor* (60% DP) and *B. adusta* MUT 2295 (55% DP). Then, DP remained essentially constant (*T. versicolor*) or increased to 76–80% (*B. adusta* MUT 2295 and *T. pubescens*). *P. spadiceum* caused an important decolourisation, up to DP yields (80%) of the best strains within 48 h. Fungal biomasses at the end of the process were colourless, indicating the absence of adsorption phenomena. No decolourisation was observed in the AS line and low values (DP < 20%) in the abiotic controls, probably due to the development of a small amount of biomass in consequence of the glucose addition in the wastewater.

Table 1
Decolourisation percentage (DP), pH, laccase (Lac), manganese-independent (MiP) and manganese-dependent (MnP) peroxidases activity (U/l) and COD values (mg/l) during W1 treatment by nine fungal strains, activated sludge (AS) and abiotic control.

	24 h					48 h					120 h					
	DP	pH	Lac	MnP	MiP	DP	pH	Lac	MnP	MiP	DP	pH	Lac	MnP	MiP	COD
<i>B. adusta</i> MUT 2295	55.4	6.2	8.3	31.9	24.3	74.7	5.4	20.6	104.0	32.9	80.5	6.0	2.8	113.3	90.2	416
<i>P. spadiceum</i> MUT 1585	43.4	6.5	4.2	11.1	3.3	81.7	4.8	1.7	63.1	12.5	76.9	5.9	1.3	87.3	72.6	658
<i>T. pubescens</i> MUT 2400	76.1	5.0	542.2	40.5	13.0	80.3	4.0	690.7	13.8	18.7	76.0	7.0	782.8	32.1	33.4	645
<i>T. versicolor</i> MUT 3403	59.9	7.6	820.0	48.2	4.4	62.1	7.6	801.1	0.0	0.0	60.4	8.5	250.4	3.8	19.8	1211
<i>C. gallica</i> MUT 3379	40.9	7.9	70.3	1.6	0.0	42.1	8.1	261.7	0.0	0.7	42.5	8.3	263.6	7.1	0.7	475
<i>S. ochraceum</i> MUT 3435	36.5	7.4	6.7	10.5	8.7	42.3	7.9	4.3	6.3	5.3	41.4	7.9	18.5	2.3	0.2	404
<i>B. adusta</i> MUT 3060	46.0	7.3	6.7	28.0	29.2	42.5	7.3	3.6	21.8	20.9	33.8	8.1	5.3	3.6	1.4	431
<i>T. hirsuta</i> MUT 3438	40.8	8.4	10.3	19.9	2.9	38.7	8.3	51.7	0.0	0.0	13.5	8.6	7.2	0.0	0.0	457
<i>G. adspersum</i> MUT 3427	4.6	7.1	11.2	0.0	0.0	0.0	7.6	122.5	0.1	-3.0	0.0	8.3	25.8	0.0	5.0	810
Activated sludge (AS)	0.0	8.4	0.9	1.2	0.0	0.0	8.5	1.7	0.0	0.0	0.0	8.3	3.9	1.5	1.2	34
Abiotic control	16.2	8.7	0.0	0.0	0.0	20.2	8.6	18.6	0.0	0.0	14.2	8.7	42.1	0.0	0.0	147

Interestingly, the strains with the highest decolourisation activity showed also the highest Lac (*T. pubescens*) and peroxidase activities (*B. adusta* MUT 2295 and *P. spadiceum*), indicating a good correlation between these enzymes and dye degradation, as already observed in a previous work (Anastasi et al., 2011) and by other authors (Diwaniyan et al., 2010). Very high Lac activities values were also produced by the strain *T. versicolor*, the fourth in terms of decolourisation efficiency (about 60% DP in 24 h).

The higher degradation efficiency of *B. adusta* MUT 2295, *T. pubescens* and *P. spadiceum* can also be correlated with the lowering of pH, which is linked to the onset of fungal metabolism (Kuhad et al., 1997). This pH value is close to the *optimum* for the fungal enzymes, that would

therefore be more active and effective as already observed in a previous work (Anastasi et al., 2010).

The COD value was slightly reduced (initial value 485 mg/l) in the lines of *B. adusta* MUT 2295 and MUT 3060, *C. gallica*, *S. ochraceum* and *T. hirsuta*; in the other cases an increase was observed, probably due to the release of metabolites and enzymes by the fungal biomass. In this regard, however, it is important to remind that the initial value (analysed before the addition of the fungal biomass) does not include the amount of COD due to the residual culture medium that could eventually remain adsorbed to the fungal biomass itself. For this reason, in the next phase, an analysis of COD at the initial stage of the experiment, just after the addition of the biomasses, was included. The AS line showed, as expected, the best results in terms of COD reduction (over 90%); the abiotic control showed a high COD reduction, again in consequence of the biomass development after glucose addition.

Hence, from the results of this first phase is clear that the fungal treatment is more effective in the removal of colour; on the other hand, the activated sludge is more effective in the reduction of COD. Thus, in the next step the applicability of a sequential treatment that takes advantage of the different potentials of these two biological components was evaluated.

Considering all these data, the best results in terms of fastness and efficiency of decolourisation were obtained by the strains *T. pubescens*, *B. adusta* MUT 2295 (that caused also a slight COD reduction) and *P. spadiceum*, that were then selected for the next phase in which the applicability of a sequential biological system was evaluated. The system consisted of a first step, performed by the selected fungal biomasses (both free, F, and supported, S), mainly devoted to the effluent decolourisation, and of a subsequent AS stage, operated to reduce the remaining COD and toxicity.

During step 1, the fungal biomasses, both free and supported, caused a rapid decolourisation of the effluent, with DP values over 60% by *T. pubescens* and *B. adusta* MUT 2295 (Table 2). These values were already reached in 24 h (data not shown). The decolourisation efficiency of AS was almost half that of fungal lines. At the beginning of step 2, because of the contribution in colour of the activated sludge, the effluent resulted more coloured (DP values reduced); then, at the end of step 2, in both *T. pubescens* lines, the DP values returned back to values around 60% (Table 2).

Table 2.

Decolourisation percentage (DP), COD (mg/l) and toxicity (growth inhibition, I%, method UNI EN ISO 8692:2005) during W2 treatment by free (F)/supported (S) fungal biomasses (step 1) and by activated sludge (step 2).

		t48 step1				t48 step2			
		DP	COD	I%		DP	COD	I%	
				Control	Sample			Control	Sample
<i>P. spadicum</i> 1585	F	58.2 ± 3.2	829.0	-3.5 ^a	16.8 ^a	48.5 ± 5.5	647.0	nd	nd
	S	47.9 ± 3.6	698.0	-3.5 ^a	54.4	57.8 ± 2.3	404.0	-3.5 ^a	44.5 ^a
<i>B. adusta</i> 2295	F	62.0 ± 3.4	389.0	12.7 ^a	51.9	52.3 ± 1.9	193.0	60.7	34.4 ^a
	S	63.8 ± 2.5	629.0	12.7 ^a	72.6	56.6 ± 2.0	358.0	nd	nd
<i>T. pubescens</i> 2400	F	62.5 ± 4.1	586.0	60.7	22.8 ^a	58.4 ± 10.1	160.0 ^a	60.7	19.9 ^a
	S	64.7 ± 3.7	497.0	60.7	23.2 ^a	66.2 ± 2.6	145.0 ^a	60.7	16.3 ^a
Activated sludge		30.2 ± 2.1	68.0 ^a	60.7	1.1 ^a				
Abio		0.0 ± 2.2	153.0 ^a	60.7	-40.8 ^a				

a Indicates that values are under the threshold limit values of the Italian law, D.L. 4/2008.

At the beginning of step 1, a Lac activity (around 140 U/l) was recorded in all culture lines; this activity, probably of bacterial origin, is present in the wastewater independently by the addition of fungal biomass, as evidenced by the values in the abiotic controls and AS line (138 and 116 U/l respectively). During step 1, this activity remained high only in fungal lines (particularly in *T. pubescens*), probably as a result of the contribution in Lac activity by the strains themselves. During step 2, the activity decreased with the exception of the line S of *T. pubescens*, where there has been a marked increase up to 1000 U/l. As regards the peroxidase activity, both MiP and MnP values remained low during the whole experiment, in all fungal lines.

The pH values do not show a lowering as in W1 experiment; on the contrary, a progressive increase of values was observed: from the initial one (pH 7.1) to 7.7–8.4 at the end of step 1 and to 8.3–8.7 at the end of step 2. These differences could be partially explained by a different composition of the effluents, although they were sampled in the same tank of the same dyeing plant. Actually, as also observed by Vanhulle and collaborators (2008), the wastewaters even coming from the same plant show large fluctuations of the main parameters as a result of different manufacturing processes.

In all lines, the addition of the fungal biomass at the beginning of step 1 caused a considerable increase in the COD values (ranging from 742 to 1504 mg/l) compared to the initial one (515 mg/l). However, as in W1 treatment, during step 1 the fungal activity caused an important reduction (from 20% to 54%) of COD values (Table 2). The AS line showed the best result in terms of COD reduction (about 80%); also in this case, in the abiotic control a high COD reduction was observed, in consequence of the biomass development after glucose addition. As expected, a further COD reduction (from 22% to 71%) was observed during the step 2 by the action of the AS. In particular, in both *T. pubescens* lines and in *B. adusta* MUT 2295 line L (Table 3), the COD reduction brought the values close to or included in the threshold limit values of the Italian law (160 mg/l, DL 4/2008).

Table 3.

Decolourisation percentage (DP), COD (mg/l) and toxicity (germination index, GI%, method UNICHIM N. 1651, 2003) during W3 treatment by *T. pubescens* MUT 2400 in bioreactor, flasks A and B and by AS line (step 1) and by the activated sludge (step 2).

	t48 step1			t48 step1		
	DP	COD	GI%	DP	COD	GI%
t0	0.0	278.0	59.0 ^a	–	–	–
Flask A	86.1	1641.0	3.0	98.2	318.0	49.7
Flask B	82.0	1665.0	4.1	85.1	338.0	18.3
Bioreactor	31.3	717.0	90.0 ^a	37.1	138.0 ^a	92.3 ^a
AS line	16.0	110.0 ^a	136.2 ^a			

a Indicates that values are under the threshold limit values of the Italian law, D.L. 4/2008.

The *P. subcapitata* analysis (Table 2) allowed to follow the evolution of toxicity during the two stages of wastewater treatment and to further emphasize the different role of the fungal and the AS culture. The fungi caused in general an increase of toxicity (higher values of I%). The only exception was *T. pubescens* that caused a significant reduction of toxicity (about 1/3 of the initial value). The AS treatment caused a toxicity reduction with only exception of the line F of *P. spadiceum*.

Considering all the parameters, the best results were obtained with the coupled treatment fungus plus AS in the line with *T. pubescens*: during step 1, the highest decolourisation was obtained and, after step 2, a consistent COD and toxicity reduction were observed, too (values within the limits of the law DL 4/2008). These data are very encouraging with respect to the biological treatment currently in place in the plant; actually, the activated sludge only (AS line) allows to obtain an efficient removal of COD and toxicity, but not an effective decolourisation of the wastewater obliging the owners to perform an ozonation treatment.

These data confirm the high dye-decolourising ability of a strain of *T. pubescens*, as already described by other authors (Enayatzamir et al., 2009 and Rodriguez-Couto, 2011) and the utility to use the complementary action of fungal and bacterial biomass in textile wastewater treatment. Actually, the fungus *T. pubescens* and the subsequent AS treatment resulted effective in respect to all parameters taken into account: colour, COD and toxicity. These data strengthen the observations of Novotny and collaborators (2011) that in a recent study demonstrated the efficacy of a similar two-step treatment, with an immobilized culture of *Irpex lacteus* used in the first decolourizing step and a mixed bacterial community for removal of organic compounds in the subsequent step. However, our system, validated on a real effluent in non sterile conditions, with shorter operation time and with toxicity monitoring, can be considered predictive of a future industrial application.

Another important advantage from a practical point of view is the fact that the *T. pubescens* strain resulted sufficiently robust and, when supported on polyurethane foam, did not show any alteration of its degradative capacity. Hence, the next step was performed with the supported biomass that represent a technological solution more advantageous from an applicative point of view. Actually, as demonstrated by other authors (Park et al., 2011), immobilized fungi are easier for liquid–solid separation, cause minimal clogging in continuous flow systems and are more resistant to mechanical stresses, pH changes or toxic exposure.

During W3 treatment (Table 3), the combined effect of the supported biomass of *T. pubescens* and of AS was tested at a higher scale. Even in presence of the same loading factor, during step 1 the fungus showed a different decolourisation efficiency in flask and in bioreactor. In both A and B flasks lines, the DP values were above 80%, comparable to previous experiments; in the bioreactor the DP was around 30%.

With regard to the COD, a strong increase of the values was observed after fungal biomass addition (this effect persists even at the end of step 1). Thus, during step 2, a strong decrease of the values was observed, in particular in the bioreactor (-65%). The flasks, on the contrary, despite a good DP, showed a lower COD reduction (about 14–20%). The only treatment with activated sludge (AS line), as in the previous experiments, resulted very effective in COD removal (-72%).

Concerning toxicity, as well as for COD, the best results were observed in the bioreactor: already in the first step by the fungal biomass, a reduction of the toxicity of the half was observed. The only treatment with activated sludge (AS line), resulted very effective in toxicity reduction, causing even a biostimulation.

From the results of this latter stage, it is clear that an optimization of the reactor technology is needed, before a full exploitation of this biological treatment potential could be achieved. Actually, the scale-up of the volumes in flask did not result in any worsening of the DP; hence it is therefore evident that the lower DP that occurred in the bioreactor was not due to the higher working volume (2 l), but to the procedural conditions of the reactor itself. The fungal treatment, however, even in bioreactor, was more efficient in removing colour than the AS, which alone removed only 16% of the colour.

With the perspective of industrial scale-up in the near future it will be important to verify the decolourisation capacity of this fungal strain towards other textile wastewaters, which, as already mentioned, are characterized by extreme variability. Moreover, an optimization of the operating parameters of the bioreactor will be performed, for example regulating the aeration and the stirring conditions, or increasing the loading factor of the fungal immobilized biomass or evaluating different types of bioreactors, in order to define the optimal conditions that ensure an efficient removal of pollutant compounds in the wastewater. Other aspects that should be addressed include the possibility to perform multiple cycles of treatment with the same fungal biomasses and the disposal of the exhausted ones.

4. Conclusions

The biological process in two phases allowed to effectively treat the wastewater under examination: an effective colour reduction was obtained by the fungal treatment and an effective reduction of COD and toxicity by the AS treatment.

The final scale-up phase in MBBR allowed to verify the effectiveness of treatment with high volumes of effluent by the immobilized fungal biomass. Despite promising results, further steps must be taken in order to optimize the use of these biomasses for a full exploitation of their oxidative potential in textile wastewater treatment.

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