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Effective Biological Treatment of Landfill Leachates by means of Selected White Rot Fungi

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In the present study, autochthonous and allochthonous fungal strains were tested towards landfill leachate. The efficacy of the treatment was monitored following the decolourisation percentage, the chemical oxygen demand and the toxicity. Among the tested strains, *Porostereum spadiceum* showed the best activity (40 % of decolourisation in one week). Fungal treatment showed a complete spectrum of action, being able also to significantly reduce the wastewaters toxicity.

1. Introduction

During past decades, both public opinion and institutions became more and more aware about the risks related to the discharge of wastewaters, which represent a serious threat to the environment. In fact, large quantities of toxic, low biodegradable and strongly coloured effluents are continuously produced and their treatment is a mandatory step (Lofrano and Brown, 2010). Biological approach by means of selected fungal strains, could offer many advantages, compensating the shortcomings shown by other processes (Kaushik and Malik, 2009). Actually, fungal extracellular oxidative enzymes with non-selective catalytical activity are the responsible agents of the degradation of recalcitrant compounds with high redox potential, which are instead hard to handle by other organisms, such as bacteria (Wesemberg et al., 2003). In particular, white rot fungi have often displayed the promising capacity to degrade aromatic compounds and to remain active in presence of harsh living conditions: both these features outline their potential application in wastewater treatment plants (Anastasi et al., 2010a; Spina et al., 2012).
These advantages could be of particular interest in the exploitation of fungi for the bioremediation of landfill leachates. Actually, leachates are principally constituted by recalcitrant and toxic xenobiotics, indicated by their low BOD/COD ratio (<0.5 mg/L), which is a limit for the growth and the metabolic activity of heterotrophic bacteria in activated sludge (Schiopu and Gavrilescu, 2010). The ineffectiveness of traditional treatments is particularly evident in the persistence of the dark colour in effluents coming out from leachate treatment plants (Primo et al., 2012).
In general, the selection of robust and versatile strains is a necessary starting point in the exploitation of fungi for wastewater bioremediation. In fact, the maintenance of non-sterile conditions for long-term operation could limit even the most powerful dye-decolorizing fungal strain. In the past years at the *Mycotheca Universitatis Taurinensis*, several white rot fungi were screened for both the effectiveness in xenobiotics degradation and the capability to grow in the presence of a very stressing environmental conditions (physical and chemical parameters, competition with bacteria) (Anastasi et al., 2012).
In the present work, one of these strains, *Porostereum spadiceum* (MUT 1585), was studied to assess its effectiveness in the decolouration of a real effluent from landfill leachate treatment plant.

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2. Materials and Methods

2.1 Real effluent

The effluent was sampled after the biological oxidation treatment of landfill leachates. It was dark coloured, with 2532 mg/L COD, 408 mg/L ammonium (as NH_4), and the initial pH was 8.5. The effluent was taken to a more acidic value (around 5) and a low glucose amount (0.1 g/L) was added. These modifications were necessary because previous experiments (data not shown) indicated that the effluent did not provide environmental condition for fungal growth. The decolourisation percentage (DP) was calculated as the percentage decrease of the absorbance spectrum in the visible range (360-790 nm) with respect to the untreated effluent spectrum (TECAN Infinite M200, Austria) (Anastasi et al., 2010b).

2.2 Fungal strain

Porostereum spadiceum (MUT 1585) was selected for its capability to degrade dyes in simulated and real textile effluents (Anastasi et al., 2012) and were kindly provided by the *Mycotheca Universitatis Taurinensis* Collection (MUT, University of Turin, Department of Life Sciences and Systems Biology).

2.3 Description of experimental set up – Biodegradation experimental

The fungus was precultured in Petri dishes contained agarised malt extract medium (MEA), containing 20 g/L glucose, 20 g/L malt extract, 20 g/L agar, 2 g/L peptone. Twenty portions (5 mm diameter) taken from the margins of the fungal colony were used as inoculum in 500 mL flasks containing 200 mL of a high nitrogen content medium, GHY (10 g/L glucose and 3.8 g/L yeast extract), as previously described (Anastasi et al., 2010b). After 7 days, the culture broth was replaced with 100 mL of effluent and the cultures were incubated for 7 days, at 25 °C and 120 rpm. Daily, a sample was collected; colour and enzymatic activity were measured. At the end of the experiment, COD and toxicity were also evaluated. Abiotic controls (without fungal inoculum) were set up and each culture condition was assayed in three biological replicates.

2.4 Enzymatic activity assay

The enzymatic activity was evaluated by means of colorimetric reactions measured by a spectrophotometer (TECAN Infinite M200, Austria). Actually, enzymes oxidize a model substrate which change its colour due to the oxidation.

Laccase activity was assayed at 25 °C, monitoring 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). The reaction, which reagents are listed in Table 1, started by the addition of the sample.

Table 1: List of reagents for laccase (Lac) activity assay

Volume	Reagent
20 µl	sample
20 µl	0.05 M ABTS (water solution)
160 µl	0.1 M sodium citrate buffer pH 3

Manganese-independent (MiP) and manganese-dependent (MnP) peroxidase activities were measured at 25 °C, monitoring 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 (Vyas et al., 1994). For MnP, 25 µM $MnSO_4$ was added to the reaction mixture. The enzymatic activity was expressed as international units (U), where one unit is the amount of enzyme that oxidizes 1 µM of substrate per minute. The reaction, which reagents are listed in Table 2, started by the addition of DMAB/MBTH.

Table 2: List of reagents for Manganese-independent (MiP) and manganese-dependent (MnP) peroxidase activity assay

Volume	Reagent	MnP	MiP	Lac ref
100 µl	sample	x	x	x
50 µl	0.001 M MBTH (soluzione acquosa)	x	x	x
25 µl	0.004 M H_2O_2	x	x	
25 µl	0.004 M $MnSO_4$	x		
700 µl	0.1 M succinate lactate buffer pH 4.5	x	x	x
100 µl	25 mM DMAB/MBTH	x	x	x

2.5 Ecotoxicological tests

The ecotoxicological analyses were carried out before and after the fungal treatment of the effluent. Two test organisms, a unicellular green alga and a dicotyledonous plant, were selected on account of their sensitivity to wastewaters toxicity (Tigini et al., 2011).

The algae tests were performed according to the standard UNI EN ISO 8692: 2005 using a monospecies culture of *Pseudokirchneriella subcapitata* (Korshikov) Hindak (ex *Selenastrum capricornutum* Prinz.). A cell suspension (2.5 10^4 cell) were inoculated in 2.5 mL of different effluent dilutions (100 %, 50 %, 25 %, 12.5 %, 6.5 %, 3.2 %, 1.6 % and 8 %). The trial was performed in triplicate. Moreover, an abiotic control (effluent without the algal inoculum) and 6-replicated biotic control (algal inoculum in water without effluent) were performed, as prescribed by the method.

After 48 h of incubation at 23 °C in the light (8000 lux), the cells concentration was spectrophotometrically measured by means the conversion from the absorbance to cell concentration thanks to a linear correspondence of this parameter previously assessed. The inhibition of the algal growth was expressed as a percentage with respect to the average of the algal growth observed in the 6 biotic controls. The results were plotted on dose-effect charts.

The dicotyledonous plant, *Lepidium sativum* L., was used for phytotoxicity tests, according to the standard method UNICHIM 1651: 2003. The seeds (90% germination warranty) were purchased from Ingegnoli S.p.A. (Milano) and were put in a Petri dish (9 cm diameter) containing 5 mL of different effluent dilutions (100 %, 50 %, 25 %, 12.5 %, 6.5 %, 3.2 %, 1.6 % and 8 %) and a paper filter (Whatman No.1) to overcome the effect due to the surface tension of the water. The trials were performed in 4 replicates. The control was performed in 4 replicates, using distilled water instead of diluted effluent.

The seeds were incubated for 72 h in the dark at 25 °C. At the end of the test, the inhibition of the root elongation was calculated as a percentage with respect to the average of the elongation observed in the 4 controls. The results were plotted on a dose-effect chart.

3. Results and discussion

3.1 Biodegradation experiment

P. spadiceum (MUT 1585) achieved a decolouration percentage up to 51 % within 7 d treatment (Figures 1 and 2). After the first hours, when probably biosorption took place reducing the colour of about 20 %, the subsequent decolouration can be ascribable to the enzymatic activity of the fungus. Actually, a peak of both decolouration (50 %) and peroxidases concentration (140 U/L) was recorded at 24-48 h from the beginning of the experiment. After this period, the decolourisation remained unchanged, while the peroxidases concentration decreased. The production of laccases was very low (Figure 1).

It is important to note that the pH of the effluent was stable for all the duration of the experiment. Thus, the fungus maintained the optimal conditions for its enzymatic activity, ensuring their stability. Actually, both laccases and peroxidases have a maximum activity in acidic environment (the optimum is around pH 4.5 - 5.5).

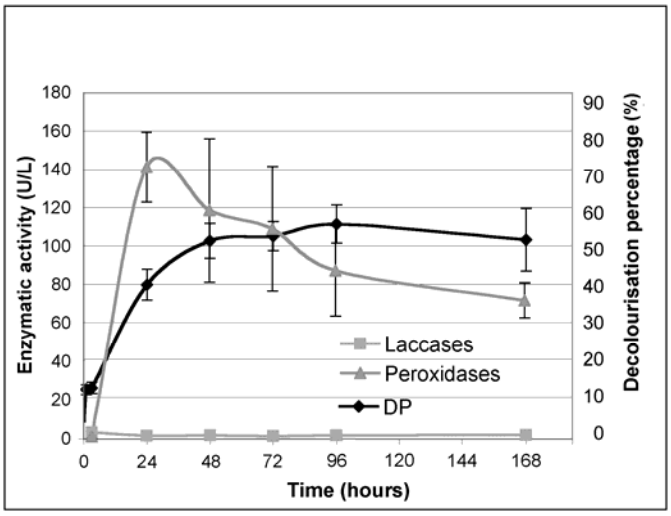

Figure 1: Fungal enzymatic activity (Laccase - Lac, Peroxidases - MnP) and decolouration percentage (DP) of the effluent during the biodegradation experiment

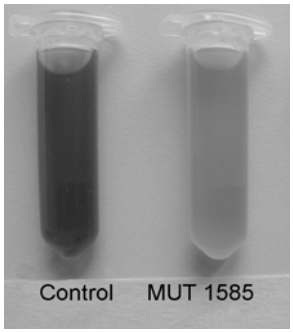

Figure 2: Visual aspect of effluent before and after fungal treatment.

Noteworthy, *P. spadiceum* (MUT 1585) was able to remain active and to compete with the microorganisms present in the effluent. Actually, from this effluent a variety of mitosporic fungi and several basidiomycetes were isolated; moreover, a lot of bacteria and unicellular algae were found (Selbmann et al., 2013). The result confirms the physiological versatility already observed for this fungal strain: actually, this fungus showed to be able to growth and remain active in harvest conditions, due to both the chemico-physical parameters and the biological competition with bacteria from activated sludge (Anastasi et al., 2012). The ability to compete with autochthonous microorganisms is a prerequisite condition for the exploitation of fungi in wastewater treatment plant, since from a practical point of view it is not possible to work in sterile condition. Moreover, from an applicative point of view, the competition with bacteria is one of the main causes that hamper the exploitation of fungi at industrial level (Anastasi et al., 2010b; Novotny et al., 2011).

3.2 Ecotoxicity tests

The results of ecotoxicity test were plotted in dose-effect charts. All the tested doses that caused a 100 % inhibition effect were discarded, because they are not usable to mark out a correct linear regression line between the two parameters.

The leachate effluent showed a very high toxicity towards both the alga and the dicotyledonous plant. In particular, *L. sativum* showed the highest sensitivity towards the sample, since the linear regression line has a very high slope and it is translated nearest ordinates axis (Figure 3).

The pH acidification decreased the toxicity towards *L. sativum*. The pH can influence metal mobility factors. Thus, the pH of the original effluent (8.5) could interfere with bioavailability of Fe ions and other fundamental micronutrients. Its decrease probably caused the solubilisation of such nutrients and allows the plant to growth. On the contrary, for the alga the effluent toxicity did not change after pH modification, even if the optimum value for this organism is around pH 8.2. Probably, in this case, the colour of the effluent strongly influenced the photosynthesis and, thus, the growth of the alga.

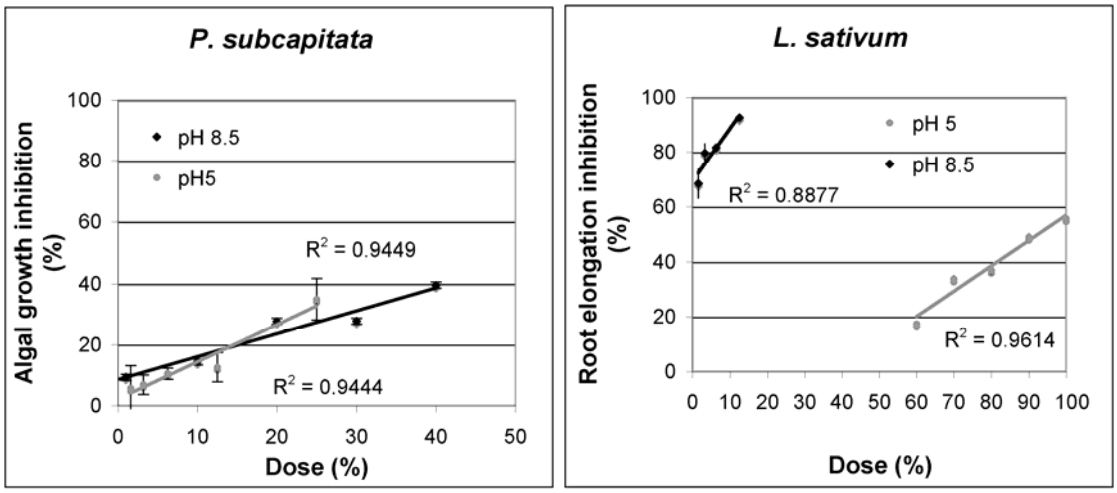

Figure 3: Ecotoxicity effect towards P. subcapitata and L. sativum caused by the untreated effluent before and after the pH modification.

The toxicity variation after the fungal treatment was expressed in percentage with respect of both the control (the effluent at pH 5) and the original effluent without pH modification (pH8.5) (Figure 4). As frequently happens, the toxicity after biodegradation experiment increased with respect to the control (effluent at pH 5). Actually, decolourisation does not imply that the molecules resulting from the degradation process are less toxic than the parent ones (Anastasi et al., 2011; Vanhulle et al., 2008).
Despite the increase of toxicity due to fungal treatment, in the case of *L. sativum* test, the comparison with the original effluent at pH 8.5 revealed, in the whole, a reduction of this parameter: the decrease of toxicity due to the effluent acidification before the fungal treatment has not completely catch up after the fungal treatment.

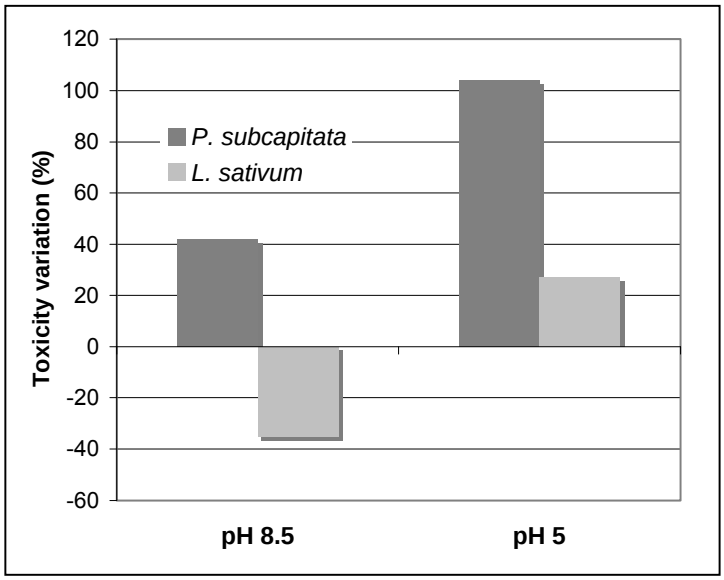

Figure 4: Variation percentage of effluent toxicity with respect of samples at different pH values.

4. Conclusions

The strains *P. spadiceum* (MUT 1585) is a powerful agent of bioremedation for coloured effluent from the treatment of landfill leachate. The effect of colour degradation resulted in the compliance of Italian law on account of wastewaters colour (not visible at the dilution 20:1). The fungus was able, not only to degrade recalcitrant coloured molecules, but also to grow in restrictive environmental conditions (competition with bacteria, presence of toxic molecules, limited concentration of easily degradable carbon sources).
This study underlined that the main catalysts involved in the degradation of coloured components of the effluent from landfill leachate were peroxidases.
Further experiments should be carried out to optimize the fungal treatment, in order to optimize the process on both sides: decolourisation and detoxification.

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