Biological treatment of industrial wastewaters: a fungal approach

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Treatment of industrial wastewaters represents an actual and spread topic. In fact, the in-use techniques are not able to completely degrade all the pollutants, present in the effluents. At the moment, other approaches are under investigation, but they often have some drawbacks in terms of economical and environmental sustainability. A strain of *Bjerkandera adusta* MUT 2295, previously selected for its capability to degrade several industrial model dyes, has been tested towards real industrial wastewaters, coming from textile and pharmaceutical industries. The efficacy of the treatment was monitored, following the decolourisation percentage (DP) and the modification of other parameters as the chemical oxygen demand (COD). The effect of the fungal immobilisation on 4 inert supports was investigated in order to select the best one in terms of biomass production and enzymatic activity. The efficiency of the immobilized biomass was assessed toward a textile effluent, comparing it with a free-cell treatment.

1. Introduction

Industrial processes are causing the production of large amount of toxic and stable pollutants, which are all collected into the water outcoming from the plant. The disposal of these contaminated effluents into receiving waters can cause environmental damages, directly influencing the aquatic ecosystem and even human being (Prigione et al., 2008).
It stands to reason that an effective treatment of these effluents is necessary. However, textile and pharmaceutical effluents are usually recalcitrant to the standard biological treatments, due to the complex aromatic compounds, the extreme chemico-physical parameters and the presence of an autochthonous bacterial microflora (Hai et al., 2008; Rosales et al., 2011). Moreover, to be competitive in the market, industries should continuously update their products, strongly influencing the industrial process itself. Consequentially, these wastewaters are very heterogeneous and complex with an inner composition which could deeply vary time by time (Vanhulle et al., 2008).
The harsh conditions which could be found in the effluent, could deeply limit even the survival of an organism. Thus, it is necessary that each new hypothesized biological treatment should combine a high efficiency with good resistance to this extreme environment. The final degradation yields could be improved by modifying the parameters which negatively influence the organism used. However when possible, the control of a specific parameter should be avoided. In fact, looking at the economical balance of the process, each operation means additional costs and more complex plant procedures.
In the past 20 years, white-rot fungi have been applied to different biotechnological fields for their capability to degrade many aromatic compounds. However, in order to investigate this fungal potential,

most of the researches have used synthetic effluents in controlled conditions. Of course, the obtained results could give little information on how a fungus could behave in a wastewater treatment plant, competing with bacterial contamination (Gao et al., 2010). To date, very few experiments have faced the industrial problematic, so that nowadays the application of fungi in a plant is still a technical challenge.

From an applicative point of view, a fungal free-cell treatment shows some drawbacks, since the mycelium could be too exposed to the environmental stresses. A good alternative could be the immobilization of the biomass on supports, with the aim to protect the biomass and improve the fungal activity (Rodriguez-Couto et al., 2009). Confirming this, it has been observed that in some cases a supported biomass showed a higher enzymatic production compared with a free one (Gao et al., 2010). Moreover, the immobilisation of the fungus could allow the use of the system repeatedly, with obvious advantages from a further application point of view.

The aim of this study was to assess if the selected strain, *Bjerkandera adusta* MUT 2295, would confirm its potential to degrade aromatic molecules, including dyes, also in a not controlled environment, as real effluents, outcoming from wastewater treatment plants. Different inert supports have been tested to select the more adapt one to host the fungal biomass. The bioremediation efficiency towards coloured wastewaters of a free-cell system and an immobilized one was compared.

2. Methods

2.1 Effluents
The wastewaters were kindly provided by Fidia Engineering S.r.l. (BG, Italy), owner of several wastewater treatment plants in Italy. The effluents were sampled from the homogenization tank before the activated sludge treatment of textile (T) and pharmaceutical (P) industries. The textile effluents (T1 and T2) were highly coloured, strongly alkaline (pH ranged between 10.4 and 11.9) and with a COD between 370 and 400 mg/L. The pharmaceutical effluent was poorly coloured, acidic (pH 4.8) and with an elevated COD (20,800 mg/L). When the pH overcame 8, it was adjusted to a standard neutral value (pH 7). When the COD was lower than 400 mg/L, a low amount of glucose (0.1 mg/L) was added to enhance fungal growth.

2.2 Organism
The strain, *Bjerkandera adusta* MUT 2295, is preserved at the *Mycotheca Universitatis Taurinensis* (University of Turin, Department of Plant Biology). The strain was selected in a previous study because of its efficient decolourisation activity towards different dye classes and effluents (Anastasi et al., 2010).

2.3 Wastewater (T1 and P1) treatment
The fungus was inoculated as twenty agar plugs (5 mm of diameter), taken from the margins of an actively growing colony on MEA, in 500 mL flasks containing 200 mL of an high nitrogen content medium as previously described (Anastasi et al. 2010). After 7 days, the culture broths were replaced with 100 mL of T1 or P1 and the cultures were followed for 5 days. In order to compare the effectiveness of the fungal treatment with the secondary treatment used in the plants, flasks containing the effluent (100 mL) and 20 mL of activated sludge, sampled in the two plants, were set up according to the procedures suggested by Fidia Engineering S.r.l. Abiotic control (without fungal inoculum) was set up and each culture condition was assayed in 3 biological replicates. The flasks were incubated at 25 °C and 120 rpm in an orbital shaker (Infors) for 5 days.

2.4 Biomass immobilization on inert supports
The experiment was carried out using 4 inert supports (Figure 1): A, circle industrial support; B net industrial support; C, polyurethane foam PUF (2 cm^3); D, stainless steel scourers (1 cm^3). Supports A and B are normally employed for activated sludge immobilization and they were kindly provided by Fidia Engineering S.r.l., while support C was kindly provided by the Department of Civil and Environmental Engineering of the University of Florence.

The fungus was pre-grown as above described (2.3). After 7 days, the biomass was harvested, homogenized and inoculated (5 mL) in flasks containing 200 mL of high nitrogen content medium and the different supports (60% of the volume). The carriers colonization was carried out both under

agitated and static conditions, in order to define the optimal colonization condition for each support. In detail, many flasks were set up for each support and in each growth condition; after 7 days, the medium was substituted with 200 mL of low nutrient content medium, in order to evaluate the fungal stability in a poor environment. Every 2 days and for 3 cycles, 2 flasks were analyzed for the biomass resilience and the enzymatic production (see below).
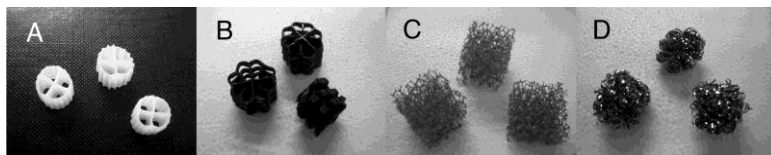

Figure 1. Inert supports used to immobilized B. adusta MUT 2295 biomass.

2.5 Wastewater (T2) treatment by free and immobilized biomass

The fungus was pre-grown as above described (2.3 and 2.4). The homogenized mycelium was inoculated in 500 mL flasks containing 200 mL of the same medium as free biomass (F) and in presence of twelve PUF (supported biomass, S). After 7 days, the culture broths were replaced with 100 mL of T2. The flasks were incubated at 25 °C in an orbital shaker at 110 rpm (F) or at 80 rpm (S) for 2 days.

2.6 Enzyme assays

Since *B. adusta* is well know to produce peroxidases (Anastasi et al., 2010), during the experiments peroxidase activity was followed. 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 (Vyas, 1994). For MnP, 25 µM $MnSO_4$ was added to the reaction mixture. The enzymatic activity was expressed as international Units (U), where 1 unit is the amount of enzyme that oxidises 1 µmol of substrate per minute.

3. Results and Discussion

3.1 Wastewater (T1 and P1) treatment

B. adusta MUT 2295 has proved to be a strong and versatile organism, able to grow in very variable and extreme conditions and effective in wastewater decolourisation and detoxification (Anastasi et al., 2010). However, its efficiency in effluents bioremediation could be still optimized, taking into account biotic and abiotic parameters, such as nutrient addition, pH values, immobilization on different supports and agitation/static growth conditions. Their optimization and their role in the bioremediation process could give useful information for the development of industrial-scale reactor (Ali et al., 2010).

In the first experiment, the fungus was tested towards 2 effluents, a textile and a pharmaceutical one, in order to evaluate if it was able to degrade aromatic molecules of different origins, facing diverse problems. The textile wastewater (T1) was highly colored and with a low COD. Since the organic compounds content was scarce, low amount of glucose was added in order to supply the fungal growth in the very early stage of the treatment. The pharmaceutical wastewater (P1), instead, was almost colorless and it was characterized by an elevated COD, up to 20,000 mg/L. It should be pointed out that all the experiments were carried out with real effluents and in non-sterile conditions, in order to evaluate whether the fungus was able to compete for carbon sources and nutrients with the autochthonous bacterial microflora (Gao et al., 2010).

Experimental data are shown in Table 1. *B. adusta* was able to remove up to 75 % of T1 colour, likely by the production of oxidoreductive enzymes as peroxidases. The relation between the active fungal metabolism and the colour reduction was also confirmed by the pH data, since it decreased from 7 to 5.4. It should be considered that many enzymes, including peroxidases, have a pH optimum among 5 and 6. It could be hypothesized that the fungus buffered the effluent in order to maintain the external environment, as more as possible close to the optimal working ones needed by its enzymes (Kaushik and Malik, 2009).

Moreover, the fungal treatment was always compared with the biological one (activated sludge) already in use in the wastewater treatment plant of interest, in order to define if the two treatments could eventually work in a synergic way. Comparing the 2 biological approaches (fungi *vs* activated sludge), the results of T1 treatment pointed out the possibility to efficiently combine them together. In fact, the fungus was effective in reducing colour but it slightly increased COD values, probably due to the release of some extracellular compounds. On the other hand, the activated sludge was almost completely ineffective in colour removal even though it was able to reduce up to 90 % of the COD. Thus, for a complete bioremediation process of textile effluents, both fungi and activated sludge seem to be fundamental, being active towards different wastewater components.

As mentioned above, since P1 was colourless, the effect of the treatment was followed by means of the COD value, only. The fungus was able to remove up to 19,000 mg/L in 5 days (90 %), resulting significantly more efficient than the activated sludge (78 %).

Table 1: Decolourisation percentage (DP), COD reduction, enzymatic production (MnP activity) and pH of T1 and P1 after the fungal and the activated sludge treatments. A negative data should be considered as an increase and not a reduction of the parameter.

	DP	COD % reduction	Perox (U/l)	pH
T1				
B. adusta	74.7	- 12	104.0	5.4
activated sludge	/	91	/	8.5
P1				
B. adusta	/	91	53.0	5.5
activated sludge	/	78	7.3	4.5

3.2 Biomass immobilization on 4 inert supports

Fungal metabolism could be greatly influenced by biotic and abiotic parameters, including the mycelium immobilization on different supports. There are many carriers available for biomass immobilization, which differ in size, structure and material. At the end, the use of a specific carrier depends strictly by the microorganism tested: it is obvious that bacterial and fungal biomass have a different biomass development, which could adapt differently to the morphology of the carriers. Furthermore, it is mandatory that the support itself does not interact with the effluent, being stable in presence of high concentration of salts and aromatic compounds, with even extremely alkaline pH. For example, alginate beads are unstable in contact with phosphate and citrate and at pH higher than 8 (Rodriguez-Couto et al., 2009 and personal not shown data). According to this, during this study, 4 inert supports have been evaluated for their capability to be colonized by the fungal mycelium in different growing conditions. Two of them (A and B) were commonly used for activated sludge colonization at industrial scale, but their potential have not been previously tested against fungi. Biomass resilience to process disturbances together with enzyme production was monitored.

As could be seen in Table 2, fungal growth was strongly influenced by the growth conditions and the support itself due to its specific structural features. The industrial carriers (A and B) did not allow an effective and persistent fungal immobilization. In fact, even if they are very efficient in bacterial biofilm formation, they seem to be not suitable for hyphal colonization. For example, on B support, fungal growth was scarce in static conditions and almost null in agitated ones. The fungus colonized the stainless steel scourers (D) in heterogeneous way and a great difference of colonization among supports could be observed. On the other hand, supports C allowed the most homogeneous and persistent biomass colonization, mainly when the support colonization was carried out in agitated condition.

Table 2: Dry weight (mg) of the biomass growth on different supports and percentage of lost biomass in comparison with the beginning. A negative data should be considered as an increase and not a reduction of the parameter.

		I cycle	II cycle	III cycle	lost %
A static	57	37	45	71	- 25
A agitated	--	--	--	--	--
B static	89	79	88	84	6
B agitated	200	132	123	111	44
C static	308	224	159	172	51
C agitated	359	325	214	203	37
D static	321	191	228	224	59
D agitated	2346	284	868	221	8

In general, looking at the enzymatic activity, when the colonization of supports is carried out in agitation, the biomass is more active: a higher concentration of peroxidases could be observed during the 3 cycles. The fungus grew on support A was able to produce a scarce or null peroxidase activity. The results obtained with the other 3 supports, colonized in agitation, are shown in Figure 2.
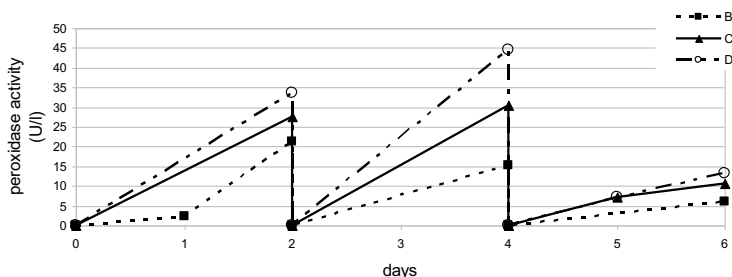

Figure 2. Peroxidase activity (U/l) produced by the fungus, grew in agitated conditions on support B, C and D, during 3 cycles.

The fungus grew on C and D supports produced the higher concentration of peroxidases, even though after 2 cycles, the enzyme production became unstable. Comparing the data of the 2nd and the 3rd cycle, the fungus reduced the peroxidase activity of about 70 %.

Considering both the biomass resilience and the enzymatic production, C and D were the most adapt supports for fungal biomass colonization. However, it should be noted that D support shows some applicative drawbacks, since it could cause scraping problems inside the reactor. Moreover, they are very heavy and the maintenance of their movement into the reactor would be very difficult and expensive. According to this, C support was taken into consideration for further experiments.

3.3 Wastewater (T2) treatment by free and supported biomass

From an applicative point of view, immobilization could avoid several problems of dispersed cells which strongly limit the further scale-up on larger volume reactor. For example, immobilized fungal cells on supports could allow a simple reuse of the biomass, an easier liquid–solid separation and avoid clogging phenomena (Rodriguez-Couto et al., 2009). Moreover, immobilized cultures often showed an increased enzymatic activity compared to free biomass and they could better resist to environmental stresses due to the extreme pH values and the presence of toxic molecules at high concentration (Rodriguez-Couto et al., 2009).

Once the best carrier has been selected, the efficiency of the immobilized fungal biomass has been compared with the free-cell one toward a real industrial effluent (T2). Experimental data are shown in Table 3. As previously observed by other authors (Rodriguez-Couto et al., 2009), an improved enzyme production is detectable when the fungus is immobilized on PUF (support C). The fungus obtained good yield of decolourisation (up to 60 %) and reduction of COD (48 %) and no significant differences

were recorded between the 2 culture conditions. In fact, the immobilized biomass was able to completely maintain the degradation yields of the free one and even slightly improve them.

Confirming what previously seen for a textile wastewater (T1), fungal treatment was more effective than activated sludge on colour reduction but not in COD reduction. In fact, the fungus removed more than the double of the effluent colour, even though concerning COD reduction, almost the opposite behaviour could be seen. Again, looking at a complete bioremediation process of textile effluents, the 2 biological approaches (fungus and activated sludge) had a complementary and not overlapping action, which could be fundamental for a further industrial application.

Table 3: Decolourisation percentage (DP), COD reduction, enzymatic production (MnP activity) and pH of T1 and P1 after the fungal and the activated sludge treatments.

	DP	COD % reduction	Perox (U/l)	pH
B. adusta				
free	62.0	47.6	1.4	8.2
immobilized	63.8	48.3	15.3	8.1
activated sludge	30.2	80.5	/	8.6

4. Conclusions

In conclusion, a very interesting fungal strain, *Bjerkandera adusta* MUT 2295, was selected for its capability to be active in bioremediation processes, acting towards several parameters, as colour and COD. A complementary approach with active sludge could be hypothesized.

From a practical point of view, in the future, it should be considered to evaluate the fungal potential also during longer treatment, carried out on several cycles, in order to mimic the industrial conditions the fungus would work in. Furthermore, the process should be scaled-up to larger volume, in order to confirm the robustness and the applicability of the system.

References

Ali H., 2010. Biodegradation of Synthetic Dyes - A Review, Water, Air, and Soil Pollution 213, 251-273.

Anastasi A., Spina F., Prigione V., Tigini V., Giansanti P., Varese G.C., 2010. Scale-up of a bioprocess for textile wastewater treatment using *Bjerkandera adusta*. Bioresource Technology 101, 3067-3075.

Gao D., Du L., Yang J., Wu W., Liang H., 2010. A critical review of the application of white rot fungus to environmental pollution control. Critical Reviews in Biotechnology 30, 70-77.

Hai F. I., Yamamoto K., Nakajima F., Fukushi K., 2008. Factors governing performance of continuous fungal reactor during non-sterile operation - The case of a membrane bioreactor treating textile wastewater. Chemosphere 74, 810-817.

Kaushik P., Malik A., 2009. Fungal dye decolourisation: recent advances and future potential. Environmental International 35, 127-141.

Prigione V., Varese G. C., Casieri L., Filipello V., 2008. Biosorption of simulated dyed effluents by inactivated fungal biomasses. Bioresource Technology 99, 3559-3567.

Rodriguez-Couto S., 2008. Dye removal by immobilised fungi. Biotechnology Advances 27, 227-235.

Rosales E., Pazos M., Sanromán M. A., 2011. Comparative efficiencies of the decolourisation of leather dyes by enzymatic and electrochemical treatments. Desalination 278, 312-317.

Vanhulle S., Trovaslet M., Enaud E., Lucas M., Taghavi S., van der Lelie D., van Aken B., Foret M., Onderwater R. C. A., Wesemberg D., Agathos S. N., Schneider Y., Corbisier A., 2008. Decolourisation, cytotoxicity and genotoxicity reduction during a combined ozonation/fungal treatment of dye-contaminated wastewater. Environmental Science Technology 42, 584-589.

Vyas B. R. M., Volc J., Sasek V., 1994. Effects of temperature on the production of manganese peroxidase and lignin peroxidase by *Phanerochaete chrysosporium*. Folia Microbiologica 39, 19-22