

# Recalcitrant Compounds Removal In Raw Leachate And Synthetic Mixtures Using *Bjerkandera adusta*

## Fungal Treatment For Recalcitrant Compounds Removal In Raw Leachate And Synthetic Mixtures

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**Abstract:** Recalcitrant compounds represent one of the major problems in wastewater treatment since biological processes, based on bacterial degradation, are not suitable for their removal. Recently, the capability of white-rot fungi (WRF) in transforming recalcitrant pollutants generated a significant interest among bio-based industries.

This study focused on the treatment of 3 effluents with the white-rot fungus *Bjerkandera adusta* MUT 2295 in batch tests. The fungal strain *B. adusta* MUT 2295 was selected during a previous decolourisation experiment due to its ability to act towards a raw leachate sample (Italy). Treatment efficiency of *B. adusta* was evaluated on a) landfill leachate deriving from Brady Road landfill in Canada and b) two solutions containing synthetic recalcitrant compounds prepared with 1) tannic and 2) humic acid. Different parameters such as the pH of the treated effluent, its chemical oxygen demand (COD) and glucose consumption of *B. adusta* during the treatment were monitored for 10 days of fungal treatment. COD removal was up to 48%, 61% and 48% in in raw leachate and the two synthetic solutions containing tannic and humic acids. Moreover, leachate color removal between 25% and 49% was achieved after 1 week of treatment. Results obtained encourage further investigations on the use of the selected white-rot fungus as potentially suitable for the treatment of the tested recalcitrant compounds.

**Keywords:** landfill leachate, recalcitrant compounds, white-rot fungi

## INTRODUCTION

The concept of recalcitrant compounds was introduced to define structurally novel and naturally occurring compounds resistant to microbial attack and persistent in the environment for extended periods (Alexander 1965). Consequently, these compounds represent a serious concern in wastewater treatment processes since the state of the art technologies for their removal are, in general, complex and not sustainable in terms of costs (Prieto-Rodríguez *et al.*, 2012; Kamaruddin *et al.*, 2014). The search for alternative, efficient and green technologies led to an increasing interest in biological processes that are typically implemented at low operating costs. In particular, within biological processes, the white-rot fungi (WRF) and their extracellular enzymes have been regarded with increasing interest in terms of hazardous and recalcitrant pollutants removal (Kalčíková *et al.*, 2014).

Pollutants biodegradation capacity of WRF is correlated with their ability to secrete extracellular enzymes such as lignin peroxidases (LiP), manganese peroxidases (MnP) or laccases (Schoemaker 1990), which are involved in lignin and lignocellulosic substrates degradation (Wesenberg *et al.*, 2003).

Due to the high organic content and complex composition, the decontamination of landfill leachate requires innovative and sustainable technologies, among which the fungal-based one shows promising result (Kamaruddin *et al.*, 2014). Treatments involving fungi may be more beneficial than those using bacteria since they offers an easier degradation of high molecular mass organic pollutants and a higher rate of COD reduction in extreme environments (Ellouze *et al.*, 2008).

55 However, at the moment, only a limited number of studies are available in the literature about the  
 56 use of WRF in landfill leachate treatment. Saetang & Babel (2012) revealed that *Trametes*  
 57 *versicolor* BCC 8725 could remove 78% color, 68% BOD<sub>5</sub> and 57% COD from leachate sample  
 58 within 15 days of incubation. Ellouze *et. al* (2008) detected COD removal efficiencies for  
 59 *Phanerochaete chrysosporium*, *Trametes trogii* and *Lentinus tigrinus* of 68%, 79% and 90%,  
 60 respectively with a two-fold dilution of leachate. COD removals were accompanied by a significant  
 61 enzyme secretion and a high reduction in the toxicity expressed as percentage of *Vibrio fischeri*  
 62 bioluminescence inhibition (% BI < 20%).

63 Data from the literature reported *Bjerkandera adusta* as effective in the degradation and  
 64 detoxification of a wide variety of wastewaters. Indeed, Anastasi *et al.* (2010) reported the  
 65 capability of *B. adusta* to completely remove the color of several dyes and three simulated  
 66 wastewaters, reducing the toxicity as well. Spina *et al.* (2012) tested *B. adusta* degradative potential  
 67 towards real industrial effluents achieving 75% color removal and 90% COD reduction in textile  
 68 and pharmaceutical wastewaters, respectively.

69 In the present study, the fungal strain *Bjerkandera adusta* (MUT 2295), previously selected for its  
 70 decolourisation capability on landfill leachate Italy, is used for the treatment of 3 effluent samples,  
 71 including raw landfill leachate (Canada) and two synthetic wastewaters containing recalcitrant  
 72 compounds prepared respectively with 1) tannic acid and 2) humic acid. The synthetic compounds  
 73 were selected as components of the recalcitrant fraction of landfill leachate (humic acid) and textile  
 74 industry wastewater (tannin). The efficiency of the treatment on the diverse recalcitrant compound  
 75 solutions was evaluated through batch tests.

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77 **METHODS**

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79 **Chemicals**

80 All chemicals used in this study were of analytical grade and purchased from VWR Canada.

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82 **Fungal strain**

83 *Bjerkandera adusta* MUT 2295 was obtained from the *Mycotheca Universitatis Taurinensis*  
 84 *Collection* (MUT), University of Turin. This fungal strain was selected out of 12 strains due to its  
 85 capability to treat raw leachates (Italy). Strain selection was performed through a biodegradation  
 86 experiment in which decolourisation potential was used as main criteria.

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88 **Target effluents**

89 The efficiency of the treatment with *B. adusta* was tested on a raw leachate collected from Brady  
 90 Road landfill (Winnipeg, Canada) and two synthetic recalcitrant compound solutions prepared with  
 91 1) tannic acid (1.3 g/l) and 2) humic acid (1.5 g/l). Details of the raw leachate chemical  
 92 characterization are reported in Table 1.

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Parameter	pH	Ammonia (mg/l)	Phosphorus (mg/l)	COD (mg/l)	BOD <sub>5</sub> (mg/l)
Value	7.61	704	1.62	1636	150

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**Table1** Chemical characterization of the Brady Road landfill leachate

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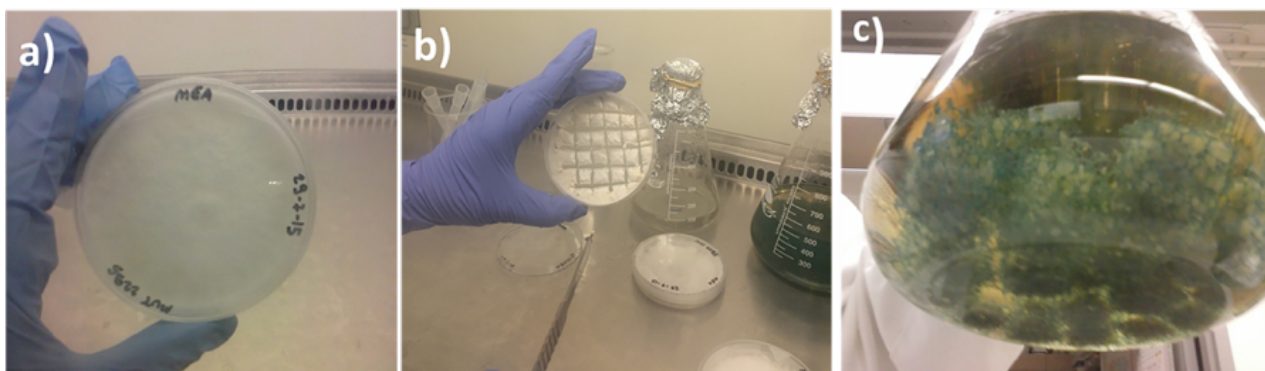
97 The two types of synthetic solutions were prepared simulating raw leachate in terms of organic  
98 load. Initial COD values ranged between 1630 and 1740 mg/l. Glucose 1 g/l was added to the three  
99 effluents as fungal co-substrate for growth. The COD values of the two synthetic wastewater after  
100 glucose addition ranged between 2545 and 2780 mg/l. The pH was adjusted to 4.5 using 10%  
101 sulfuric acid.

102

### 103 **Fungal cultivation**

104 *B. adusta* was cultured on Malt Extract Agar (MEA, 20 g/l glucose, 20 g/l malt extract, 20 g/l agar, 2  
105 g/l peptone) at 25°C for one week (Figure 1a). After the cultivation, *B. adusta* was homogenized  
106 under sterile conditions, with sterile saline (9 g/l NaCl) ( and inoculated into 1L flasks containing  
107 glucose and yeast extract liquid media (GLY, 5 g/l glucose; 1.9 g/l yeast extract) and 2 cm<sup>3</sup>  
108 polyurethane foam cubes (PUF). Flasks were incubated in agitation for one week in order to enable  
109 the immobilization of the fungus into the cubes (Figure 1c). After 7 days, the cubes were removed  
110 and added to 500 ml flasks containing target recalcitrant compounds.

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114 **Figure 1** Fungal cultivation and immobilization on PUF

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### 115 **Parameters for the evaluation of treatments efficiency**

116 A panel of parameters was used to evaluate the efficiency of the treatment. In particular, effluent  
117 pH, COD removal, decolourisation, glucose consumption, enzymatic activities (MnP and laccases)  
118 were measured for 10 days at 24 h intervals. Trials were triplicated including the same number of  
119 unseeded controls, without fungal inoculum.

120 COD was measured according to Standard Methods for Examination of Water and Wastewater 20<sup>th</sup>  
121 edition Section 5220, Hach Spectrophotometric procedure (DR2800 manual). The decolourisation  
122 percentage (DP) in 1) raw leachate and 2) humic acid solution was determined  
123 spectrophotometrically as the decrease of the spectrum area in the visible range (380-760 nm) with  
124 respect to the abiotic control. Decolourisation in tannic acid solution was measured  
125 spectrophotometrically as the decrease of the spectrum area in UV range (200-380 nm) with respect  
126 to the abiotic control. Glucose consumption was measured according to the reducing sugars  
127 protocol (Miller 1959).

128 MnP activity was measured at 25 °C, monitoring the oxidation at 590 nm of dimethylaminobenzoic  
129 acid/3-methyl-2-benzothiazoline hydrazone hydrochloride (DMAB/MBTH), in 0.1 M succinate  
130 lactate buffer pH 4.5 (Vyas *et al.*, 1994). Laccase activity was assayed at 25 °C, monitoring the  
131 oxidation at 420 nm of 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), in 0.1 M  
132 sodium citrate buffer, pH 3 (Niku-Paavola *et al.*, 1988).

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### 134 **Statistical analysis**

135 All the data were elaborated with the aid of one-way ANOVA, and the means were separated by  
136 Bonferroni multiple-comparison test ( $P \leq 0.05$ ) using the specific software Statgraphics 6.1  
137 (Statistical Graphics Corp., USA).

138 **RESULTS AND DISCUSSION**

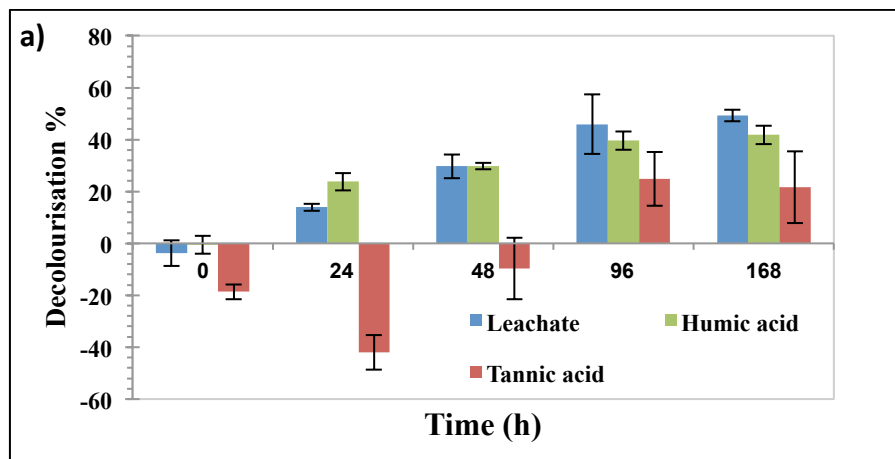
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140 **Color removal**

141 *B.adusta* decolorized raw leachate, humic acid and tannic acid solution up to 49%, 42% and 25%,  
142 respectively. Decolourisation results of one week of fungal treatment are showed in Figure 2.

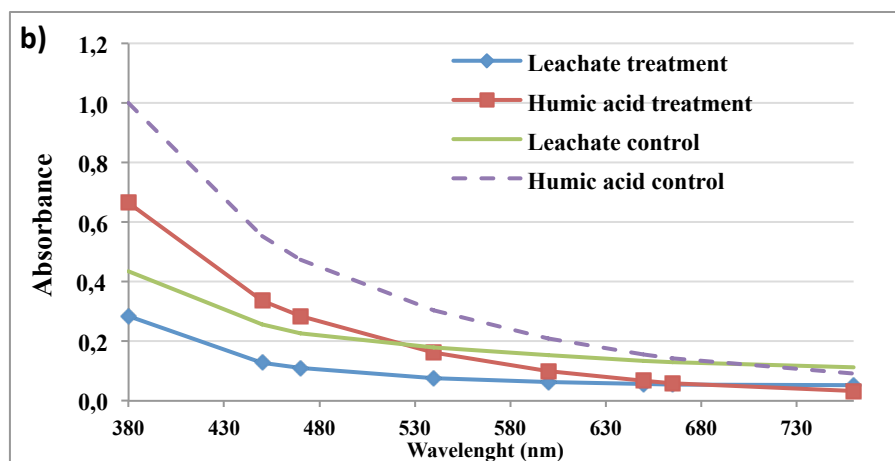
143 In the case of raw leachate and humic acid solution, it is important to notice that these effluents  
144 were dark colored and that the experiment was performed without diluting the samples. Figure 2b  
145 showed the spectrum area reduction of the two dark colored effluents towards the respective  
146 unseeded controls after one week of treatment.

147 On the contrary, tannic acid solution was initially almost colorless. After a first increase in the  
148 spectrum area within the first 48 hours of treatment, in the following days of the experiment, a  
149 reduction up to 25% was observed. In Figure 2b the spectra of the trials of tannic acid solution  
150 inoculated with *B. adusta* and the respective abiotic controls are showed. In this case, the reduction  
151 of the spectrum area and the evident flattening of the treatments spectrum shape were presumably  
152 related to a degradative process operated by *B. adusta*.

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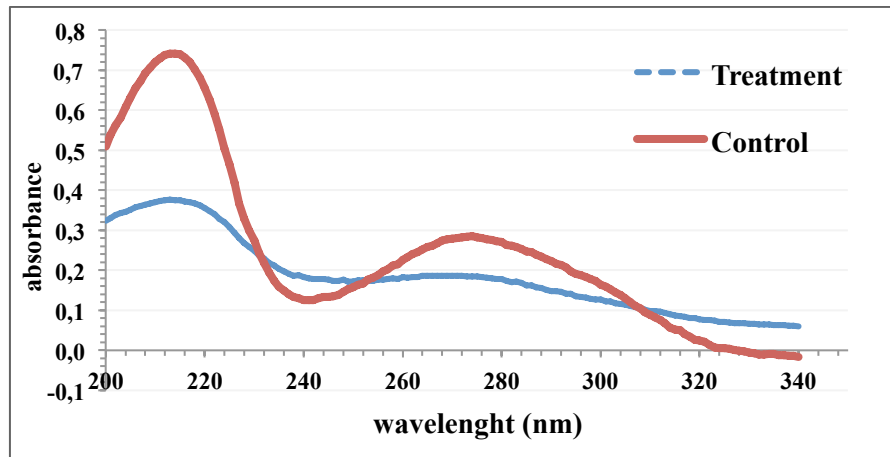


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**Figure 2** a) Effluents decolourisation percentages in one week of treatment.

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b) Spectrum area reduction in raw leachate and humic acid after 168 h and c) spectrum area

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reduction in tannic acid after 96 h. Decolourisation values are given as the average among

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triplicates with DS (+/-). A negative data should be considered as an increase and not a reduction of the parameter.

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### ***COD removal***

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Results of COD removal in the three effluents are reported in Figure 3a. Data showed a reduction of 48%, 61% and 48% in raw leachate, tannic acid and humic acid, respectively, in 10 days of fungal treatment.

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As glucose represented about 36-39% of effluent's COD, assuming its complete depletion, the detected COD removal percentages were higher than glucose consumption in all the samples inoculated with *B. adusta*. It is possible to observe a clear pattern in the two synthetic solutions where the difference in COD removal between treatments and the respective unseeded controls reached 56% and 33% in tannic acid and humic acid solution, respectively.

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On the contrary, the percentage of COD removal in raw leachate was the same in treatments and controls. This result could be related to the presence of autochthonous microorganisms capable of removing a certain amount of recalcitrant compounds from the leachate itself (Anastasi *et al.* 2010). Although there was a lack of evident COD decrease in raw leachate attributable to the presence of the fungus, the treatment could have led to different rearrangement of recalcitrant compounds chemical structure, enhancing their bioavailability for other organisms. Hence additional parameters as biological oxygen demand (BOD) could provide a deeper understanding of the process occurred during fungal treatment.

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The maximum COD removal was achieved in tannic acid, resulting in a final COD value of 995 mg/l, much lower than the initial one of 2780 mg/l, providing further evidence of the presence of a degradative process due to the treatment and confirming the results of the spectrum analysis (Figure 3b).

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The decrease of COD detected in humic acid solution treatment was presumably due to glucose consumption since the difference in the removal between treatments and controls (33%) is lower compared to the organic load represented by glucose. However, as previously hypothesized concerning raw leachate, possible rearrangements of recalcitrant compounds chemical structure due to the treatment with *B. adusta* can not be excluded.

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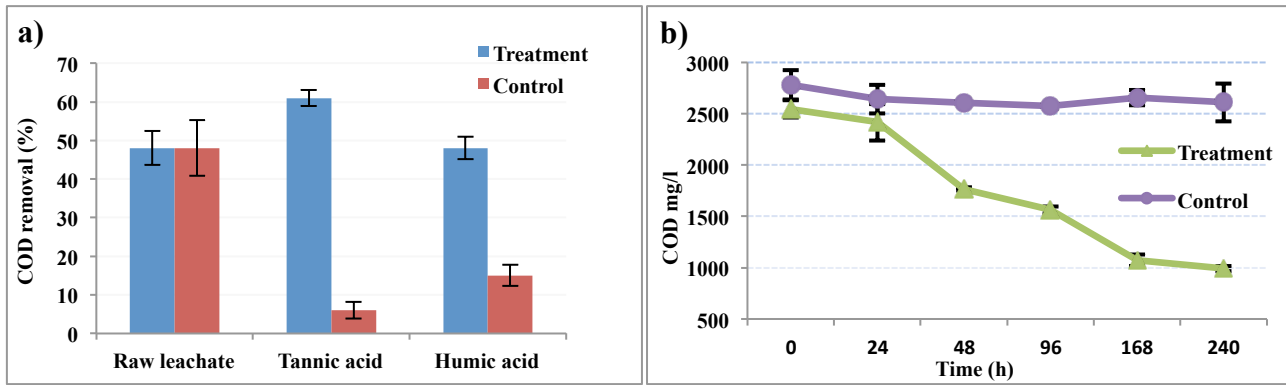
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**Figure 3** a) COD removal percentages in the effluents and b) COD removal in tannic acid as mg/l. Values are given as the average among triplicates (+/-) DS.

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### 204 *Enzymatic activities*

205 Further evidence of the presence of a degradative process related to the presence of *B. adusta* has  
206 been provided by the results of the enzymatic activities (data not shown). Indeed MnP activity was  
207 detected in all the treatments, confirming that *B. adusta* was metabolically active for all the duration  
208 of the experiment.

209 The maximum value was reached in the tannic acid solution after 72 hours with  $8.9 \pm 2.3$  U/l, in  
210 correspondence to the beginning of spectrum area reduction. The maximum values of MnP  
211 achieved in raw leachate and humic acid were respectively  $1.92 \pm 1.4$  U/l and  $5.6 \pm 1.5$  U/l.

212 These results confirm the vitality of *B. adusta* in the growth condition reported and are positively  
213 correlated to COD and color removals observed. In fact previous studies reported peroxidases as the  
214 major enzymes involved in the decolourisation of leachate (Tigini *et al.*, 2013).

215 Laccases activity was not detected in the tested conditions. This result is consistent with literature  
216 data since *B. adusta* is well known to produce peroxidases (Anastasi *et al.*, 2010).

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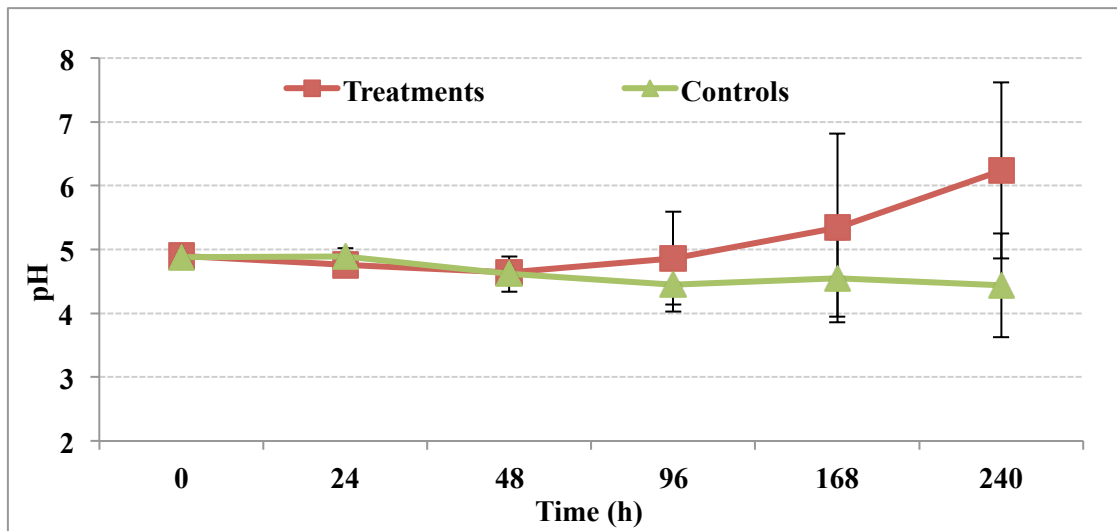
### 218 *pH values*

219 In the trials inoculated with *B. adusta*, effluents pH values ranged between 4.6 and 6.2. In the  
220 unseeded controls, the pH was between 4.4 and 4.9 (Figure4).

221 Since many enzymes, including peroxidases, have a pH optimum among 5 and 6, the values  
222 observed in the treatments were compatible with fungal active metabolism.

223 The increasing trend of treatments pH values could suggest that *B. adusta* buffered the effluents as  
224 similar as possible to the optimum enzymatic range of the enzymes involved in the process as has  
225 already been reported by Kaushik & Malik (2009).

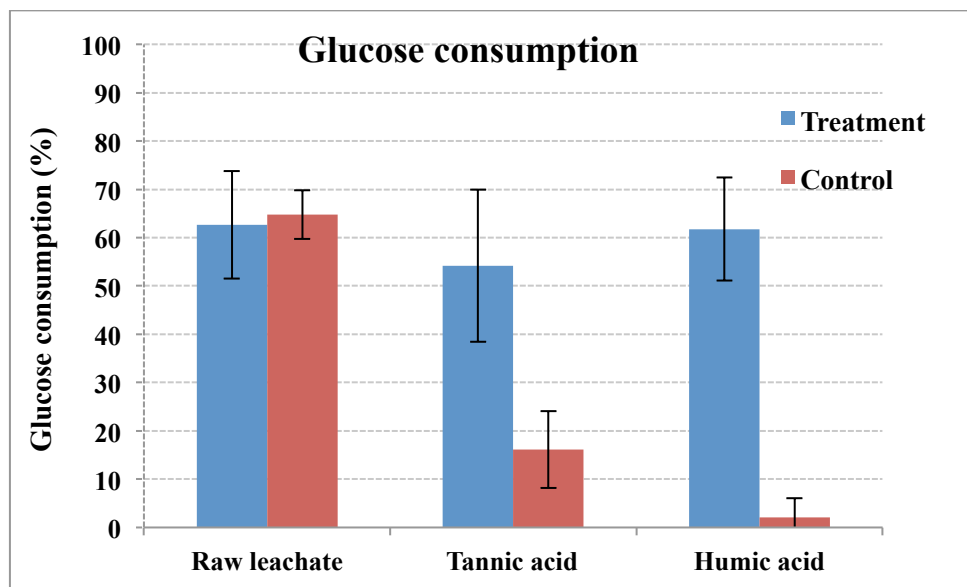
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228 **Figure 4** Effluents pH values during the treatment given as the average among trials with  
229 standard deviations (DS) (+/-)  
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231 **Glucose consumption**

232 Glucose consumption percentages detected in the treatment ranged between 54 and 63% (Figure5).  
233 Results showed low percentages of glucose consumption in both synthetic solutions controls,  
234 resulting in 2 and 13% in humic acid and tannic acid solutions, respectively. On the contrary, 65%  
235 of glucose consumption was detected in raw leachate control, suggesting the presence of  
236 autochthonous microorganisms capable of using glucose as carbon source.  
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240 **Figure 5** Glucose consumption measured against the different effluents in one week of treatment.  
241 Values are given as the average among triplicates with DS (+/-).  
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243 **CONCLUSION**

244 *Bjerkandera adusta* MUT2295 was able to survive for the duration of the experiment, overcoming  
245 the harsh conditions in terms of toxicity and autochthonous microorganisms' competition of the  
246 tested recalcitrant compounds solutions. The treatment with the selected strain resulted in the

247 decrease of several parameters such as COD and color. The process, positively correlated with the  
248 quantification of enzymatic activity (MnP), that confirmed the active metabolism of *B. adusta*.  
249 These results encourage further evaluation of the selected fungus for the treatment of the tested  
250 recalcitrant compounds. A panel of analysis would provide a deeper understanding of the  
251 degradative mechanism performed by *B.adusta*, confirming or rejecting preliminary results  
252 achieved with this strain.

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