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Fungal and enzymatic remediation of a wine lees and five wine-related distillery wastewaters

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Abstract

The aim of this work was to characterise wine-related wastewaters and ascertain the wastewater concentrations that were optimal for treatment by *Trametes pubescens*. Laccase production was also monitored. Crudely purified laccase was tested independently to determine its role in phenolic compounds degradation and colour change. The fungal treatment resulted in decreases in the wastewater chemical oxygen demand of up to 83 ± 2.1%, phenolic compounds of 87 ± 1.6% and colour of 88 ± 4.7%. Although laccase treatment lowered total phenolics by up to 61 ± 0.5%, the colour was increased by up to 160 ± 5%, indicating the formation of colour-rich compounds. *Trametes pubescens* MB 89 greatly improved the quality of all six wastewaters tested, although two wastewaters had to be diluted to below 50% to allow for bioremediation by the submerged fungal culture. Laccase synthesis greater than 1500 U/l was obtained in all wastewaters, with a maximum of 8997 U/l. The complete fungal system was found to be superior to enzymatic treatment alone. Enzymatic treatment reduced the total phenols but did little to improve the colour of the wastewaters, and in the case of wine lees significantly increased the colour.

Keywords: COD; Effluent; Laccase; *Trametes*; Vinasse

1. Introduction

The alcohol fermentation industry is divided into three main categories: brewing, distilling and wine manufacture. Each of these categories produces wastewaters with common characteristics, such as acidic pH values and high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Thassitou and Arvanitoyannis, 2001). Major difficulties in treating these wastewaters biologically include their variations in the concentration and type of organic compounds, flow volumes and inorganic constituents. A variety of wastewaters may be produced from distilleries using wine-related feedstocks. Various low-grade wines may be distilled to remove the ethanol fraction. Red wine may be distilled to increase the concentration of ethanol and volatile organic compounds to produce brandy, resulting in a wastewater commonly known as rebate, which derived from the base wine used in the production of brandy that is known as rebate wine. Wine lees is the sediment or deposit that forms in the bottom of wine casks during the fermentation process and is rich in organic constituents. Wine lees contains tartaric acid and tartrates that can be extracted, after which the ethanol content may be removed by distillation. The colour of wine-distillery wastewater is generally attributed to phenolic compounds, although in some distillery wastewaters, such as those derived from molasses, the brown colour is attributed to nitrogenous polymers known as melanoidins. These complexes form as a result of condensation reactions between reducing saccharides and amines or amino acids, which is known as the Maillard reaction (Cämmerer and Kroh, 1995).

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Distillery and wine-related wastewaters contain high concentrations of phenolic compounds, with values ranging from 29 to 766 mg/l (Bustamante et al., 2005). Certain phenolic compounds have been implicated in the inhibition of biological treatment by anaerobic digesters (Borja et al., 1993a,b). A review by Coulibaly et al. (2003) summarised a number of wastewater characteristics that could adversely affect a biological treatment process. These include temperature, pH, salts, inhibitory molecules (sulphur compounds, surfactants, heavy metals, bleaching chemicals) and the lack of a sufficient carbon and nutrient supply in raw wastewaters. Pretreatment by aerobic degradation of these wastewaters may aid and even improve a second biological treatment step. Mayer (1991) compared biological treatment of a brewery's wastewaters and found that an aerobic system would be best suited as a pretreatment step in a remediation process, as it is capable of high COD removal efficiencies under high loading rates.

White rot fungi produce enzymes such as lignin peroxidase, manganese peroxidase and laccase and are capable of completely mineralising lignin. This wood-degrading potential of fungi has allowed for the treatment of high strength phenolic wastewaters. One of these enzymes, laccase, is particularly attractive as a bioremediation tool because it has a broad substrate range, high catalytic constants and the secondary substrate, molecular oxygen, is reduced to water during the reaction (Thurston, 1994). Laccase has shown remediation potential in food industry wastewaters generated by beer production, olive milling and distilling, and may be used to stabilise wines, beers and fruit juices. These and numerous other potential applications of laccase in the food industry are described in a review by Minussi et al. (2002). Applications to delignification, ethanol production, biosensors, dye, herbicide, trichlorophenol and alkene degradation are discussed in another review, by Mayer and Staples (2002).

Although the final aim of wastewater treatment is to produce an environmentally benign discharge, a number of biologically derived products may be generated from the treatment process. This can prove advantageous in that, while simultaneously treating a waste, a product such as biogas, proteins or polysaccharides may be derived to offset the costs or energy associated with treatment. Various organic compounds in the wastewaters may be utilized as a growth source for the production of biomass or valuable compounds from biomass metabolism, such as lipids (Yamasaki et al., 2006), proteins (Morimura et al., 1994a) or enzymes (Morimura et al., 1994b). The synthesis of laccase represents a potential valuable byproduct from a wastewater treatment process using white rot fungi which may offset the costs associated with wastewater treatment. Laccase synthesis is dependent on a number of factors, including the nature and composition of the culture medium (constituents that provide a nitrogen and carbon source), type of culture conditions (oxygen availability, pH of the medium and temperature of cultivation) as well as the presence of laccase-inducing compounds.

The objectives of this study were to investigate the use of *Trametes pubescens* to lower the COD and total phenol concentrations, to increase the pH and to decrease the colour of a variety of wine-related wastewaters. Laccase synthesis was monitored concurrent to bioremediation. The effects of crudely purified laccase were assessed to determine whether it alone would be effective to treat wine-related wastewaters, independently of the fungus.

2. Methods

2.1. Culture maintenance and wastewater samples

Trametes pubescens MB 89 was purchased from Centraalbureau voor Schimmelcultures (The Netherlands, culture 696.94). The specimen was routinely subcultured on bacteriological agar (12 g/l, Biolab, Merck Chemicals (Pty) Ltd, Johannesburg) plates containing 2% malt extract (Biolab, Merck), 1% glucose (uniLAB, Saarchem, Merck) and 0.2% yeast extract (Biolab, Merck). Five distillery wastewaters were collected near to Worcester in South Africa in March and May 2006. Two of the distillery wastewaters were obtained from brandy distillation (B1 and B2) and two from spirits distillation (removal of the ethanol fraction) using low-grade wine (S1 and S2). The fifth distillery wastewater was generated from wine lees that had had its tartaric acid extracted and its ethanol removed by distillation (DL). The sixth wastewater tested in this chapter was a wine lees (L) that was obtained from a winery near to Stellenbosch in March 2006. All wastewater samples were stored at 4 °C.

2.2. Flask cultures

The pH of the wastewaters was adjusted to 4.5 using Na_2CO_3 (Saarchem, Merck). Aliquots of 65 ml were placed in 250 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and autoclaved. The wine lees samples were autoclaved individually in tightly sealed Schott bottles to prevent loss of volatile compounds, and then aseptically transferred to sterilised flasks. Distilled water containing 5 mM succinic acid (Saarchem, Merck)/lactic acid (Saarchem, Merck) at pH 4.5 served as the control flasks. Flasks were inoculated with *T. pubescens* MB 89 (0.082 ± 0.01 g dry mass) from stock cultures that had been cultured in a 10% dilution of a distillery wastewater (sample S1) containing 2% malt extract, 1% glucose and 0.2% yeast extract (all Merck, as in Section 2.1). The samples were placed in a shaking incubator (Labcon) at 150 rpm at 28 °C for 12 days. Wastewater sample flasks were conducted in triplicate. Subsamples were taken daily from each flask and centrifuged in 1.5 ml Eppendorf containers at 9660 g for 2 min (Heraeus Biofuge, Germany). The supernatant was aspirated, diluted appropriately and tested for

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laccase activity, total phenolic compounds concentration, COD, pH, and colour as indicated by a change in absorbance at 525 nm using methods described in Section 2.4. Fungal dry mass and suspended solids masses were determined using Standard Method 2540D (APHA et al., 1998).

2.3. Laccase purification and testing

Laccase was produced in a shake-flask culture in brandy distillery wastewater (sample B1) at pH 5.0 at 28 °C shaken at 150 rpm. After 10 days the biomass was removed by centrifugation and filtration. Laccase was precipitated at 4 °C by saturation with ammonium sulphate (Saarchem, Merck). The precipitate was dialysed in 1 mM succinic acid/lactic acid at pH 4.5 over a three day period, with 12-h changing of dialysis water. Dialysed materials were combined, centrifuged and filtered through Whatman no. 1 filter paper. The light yellow filtrate obtained had a laccase activity of 4200 U/l. Duplicate Erlenmeyer flasks (250 ml) containing 65 ml of wastewaters at various concentrations at pH 4.5 were spiked such that the final laccase concentration was 25 U/l. These flasks were incubated at 28 °C on a shaker (Labcon) at 150 rpm for 48 h. Subsamples were taken after 3, 6, 24 and 48 h and tested for total phenolic compounds, colour and pH.

2.4. Analytical methods

The methods for COD concentration, laccase activity and total phenolic compounds concentration determination have been detailed in Strong and Burgess (2007). The colour was measured by mixing a 150 μl sample with 150 μl phosphate buffered saline (pH 7) such that the final concentration was 20 mM and measured at an absorbance at 525 nm (PowerWave$_x$, Bio-Tek Instruments Inc, Winooska, VT, USA). Values were converted to a percentage of the raw wastewater at pH 4.5.

Dissolved iron, copper, lead, cadmium and tin concentrations were measured using atomic absorption spectrophotometry (AAS) (GBC 909 AA, GBC, Australia). Full strength wastewaters were acidified with hydrochloric acid (Merck), filtered (0.22 μm nylon filters, Micron Separations Inc), using apparatus that had been acid washed and thoroughly rinsed in deionised water. Standard curves were obtained using appropriate dilutions of 1000 ppm AAS standard solutions of the metals in 1 N nitric acid (EC Lab Services, Port Elizabeth).

The CV and DPV results were obtained using an Autolab PGSTAT 30. A 3 mm glassy carbon electrode (GCE) was used as the working electrode, while an Ag/AgCl electrode (3 M KCl) served as a reference electrode. Platinum wire was used as the auxiliary electrode. All CVs were performed at a scan rate of 50 mV/s. The GCE was cleaned thoroughly before use and between scans by polishing on a Buehler pad with alumina, rinsing with deionised water, rinsing with dilute HNO_3 and finally rinsing with deionised water again. The electrochemistry was performed in freshly prepared Britton–Robinson buffer at pH 4 (adjusted using 2 M NaOH). Britton–Robinson buffer (5 ml) was pipetted into the electrochemical cell and a scan was performed to obtain the base-line. The GCE was removed and cleaned, 20 μl of sample was added and another scan was performed in the stirred solution. A peak was observed if any electroactive compounds were present.

3. Results and discussion

3.1. Wastewater characterisation

The results for the characterisation tests are shown in Table 1. The distillery wastewaters varied in COD concentrations from 10.5 to 45.5 g/l, while total phenolic compounds concentrations ranged from 35 to 540 mg/l. All but one of the pH values were below 4. No cadmium or tin were found in any of the samples. Copper was found in relatively high concentrations in the two wastewaters from the brandy distillery (22 mg/l in B1 and 15 mg/l in B2). Iron was found in brandy distillery wastewater B2 at 14.5 mg/l and in the distilled wine lees that had had its tartaric acid extracted (DL) at 6.7 mg/l. When the distillery wastewaters tested here are compared to a wastewater derived from molasses spent wash (MSW) they have similar pH values (between 4.0 and 4.3), but the MSW had a much higher suspended solids (2.0–2.5 g/l) and COD (92–100 g/l) and a BOD of only 52–58 g/l (Nandy et al., 2002). A much higher proportion of the COD of the wastewaters treated in this study was digestible biologically (shown later). The wine lees had an extremely high total phenolic compounds concentration (1720 mg/l) and COD (212 g/l). A large portion of the COD was attributable to ethanol. Wine lees had the lowest concentrations of all metals. This could be ascribed to the fact that it was the only non-distillery wastewater, and was not heated to boiling point in metal stills or pumped at high temperatures through metal piping.

3.2. Wastewater pH

The pH is one of the fundamental attributes of a wastewater that affects its biological remediation. In biological systems an extreme pH value will prevent treatment with

Table 1
Results for characterisation tests performed on six wine industry-related wastewaters

	Fe (mg/l)	Cu (mg/l)	Pb (mg/l)	pH	COD (g/l)	Colour (visual)	Total phenols (mg/l)
B1	1.1	21.9	0.15	3.75	29.5	Dark yellow	280
B2	14.5	15.5	0.07	3.90	10.5	Yellow	35
S1	2.8	5.6	0.18	3.67	19.9	Red	320
S2	3.0	0.2	0.11	3.58	34.8	Red	290
DL	6.7	1.7	0.24	5.09	45.5	Brown	540
L	1.0	0.1	0.00	3.72	211.8	Purple	1720

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both aerobic and anaerobic processes. Even physicochemical processes are highly reliant on a particular pH for greatest effectiveness. White-rot fungi are more able to treat wastewaters under slightly acidic conditions than their bacterial counterparts, such as the methanogenic bacteria, which have an optimal pH range between 7.0 and 7.5. The pH values of all the wastewaters tested were adjusted to 4.5 to enable a comparison between the enzyme and the fungal culture by using a pH at which the crudely purified enzyme would still have high catalytic activity and the growth of the fungal culture would not be too inhibited. Optimal activity for laccase from *T. pubescens* MB 89 has been shown to vary from 3.0 to 4.5, depending on the substrates oxidised, and generally has bell-shaped curves when relating activity to pH (Galhaup et al., 2002a). The pH of all wastewaters, at all concentrations tested, increased as a result of fungal treatment (Table 2), probably as a result of the degradation of the various organic acids present in these wastewaters. A clear trend was observed: less concentrated wastewaters had greater increases in pH after treatment, demonstrating the buffering effects of compounds present in the wastewaters. The wastewaters that were treated by laccase alone displayed no change in pH.

3.3. Chemical oxygen demand removal

Initial experiments were conducted to determine the wastewater concentrations that were not toxic to *T. pubescens* by testing shake-flask cultures in 100%, 75%, 50% and 25% concentrations of all wastewaters (data not shown). With wine-related distillery wastewaters the COD and colour removal are crucial to bioremediation. The high proportion of easily degradable components in the wastewater can lead to eutrophication of receiving waters if released untreated. High salt concentrations can decrease the availability of oxygen to aerobic organisms as well as affect the osmocity. The COD results for fungal treatment after twelve days are shown in Fig. 1. The two brandy distillery wastewaters (samples B1 and B2) and one of the spirits distillation wastewaters (S1) were easily treated at full strength and had COD removal efficiencies in excess of 70%. This is comparable to results obtained by González Benito et al. (1997), who conducted laboratory batch tests to determine the ability of *Trametes versicolor* to treat molasses-based distillery wastewater. Conditions such as pH, nutrients and carbon source concentrations were tested in order to establish their relation to COD, colour and ammonium removal. González Benito et al. (1997) obtained 77% COD removal with additional sucrose and KH_2PO_4. One wastewater sample from spirits distillation (S1) was easily treatable at 100% strength, while the other proved inhibitory at 75% and 100% strength (S2). A component of the COD may have inhibited the fungal culture, as the raw COD value was 57% higher for wastewater S2 than S1. The results here compare well to results obtained by Jiménez et al. (2003) who studied the effectiveness of three *Penicillium* spp. and *Aspergillus niger* at treating beet molasses alcoholic fermentation wastewater. The four fungi they tested removed a maximum of 52% of the COD.

Two of the wastewaters (DL and L) had to be diluted to below 50% to allow for fungal remediation. The wine lees displayed no COD removal at 50% strength, but was very well degraded at concentrations of 40% or less. This may have been attributable to high concentrations of electroactive phenolic compounds, which even at a 50% concentration were considerably higher than in any of the distillery wastewaters (Table 3). Phenolic acids have been shown to affect fungal growth rates. Fitzgibbon et al. (1998) observed vanillic acid to affect the growth of *Trametes versicolor*, *Phanerochaete chrysosporium*, *Mycelia sterilia* and *Geotrichum candidum* to varying extents. Gallic acid did not affect *G. candidum* growth rates, but affected the growth rate of *T. versicolor*, *P. chrysosporium* and *M. sterilia*. In the wine lees growth inhibition may have been the result of ethanol in the wastewater, as ethanol has been shown to inhibit growth of *Pycnoporus cinnabarinus*. Fungal radial growth was halved when 20 g/l ethanol was

Table 2
Change in pH values after 12 days of fungal treatment (initial pH value 4.50, $n = 3$ and standard deviation <0.21 pH unit)

Samples and dilutions	Final pH
B1 100	6.16
B2 100	6.68
S1 100	5.27
S1 75	5.2
S1 50	5.18
S2 100	4.62
S2 75	4.66
S2 50	5.79
DL 40	4.55
DL 30	6.67
DL 20	6.96
L 40	5.44
L 30	6.15
L 20	6.45
Control	7.2

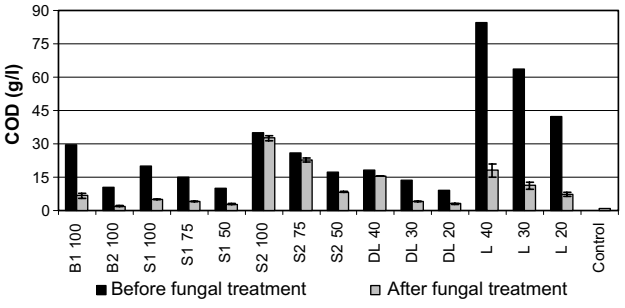

Fig. 1. Wastewater COD values before and after fungal treatment for the various wastewaters and the percentage concentration assessed at. Error bars represent standard deviation and in some cases are too small to be visible ($n = 3$).

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Table 3
Results from differential pulse voltammetry and cyclic voltammetry in relation to total phenols

WW (total phenols)	CV vs. Ag/AgCl	DPV vs. Ag/AgCl
B1 (390 mg/l)	A small peak occurs at 0.4 V	Small peaks at 0.38 V and at 0.8 V indicating low concentrations of electro-active compounds
B2 (35 mg/l)	A small peak occurs at 0.4 V	Peaks at 0.38 V and one at 0.68 V, but at very low concentrations
S1 (320 mg/l)	There are two peaks, indicating two electro-active compound present in this sample	There is a highly electro-active compound at 0.37 V. Another peak at 0.8 V is only detectable at higher concentrations
S2 (220 mg/l)	Small peak at 0.395 V	Small peak at 0.38 V. More than one compound (due to the width and the bump on the right of the peak)
DL (540 mg/l)	The lack of any distinguishable peaks indicates no electro-active compounds were present	There is an electro-active compound 0.8 V but it is only evident at higher concentrations
L (1720 mg/l)	Major peak at about 0.4 V and high concentrations of electro-active compounds at higher potentials	Very broad peak at 0.38 V. This sample contained highly electro-active compounds

added to the agar culture medium and at 30–50 g/l fungal growth was inhibited for 2–10 days and the subsequent radial growth rate was decreased by 50–70% (Lomascolo et al., 2003). At lower concentrations ethanol can prove advantageous to fungal treatment systems, as it can serve as a carbon source and an enzyme inducer. Ethanol at a concentration of 3.5 g/l enhanced laccase synthesis tremendously with *P. cinnabarinus* in the study by Lomascolo et al. (2003).

Distilled wine lees (DL) wastewater proved to be the most difficult to treat and an adequate COD decrease was only achieved when the wastewater was diluted to 30% strength. A variety of potential factors could have affected the fungus' ability to degrade the wastewaters and these have been described in the introduction to this chapter. The most probable reasons for fungal inhibition were related to toxic concentrations of components of the COD or possibly inorganic ion effects. Different fungal genera have differing tolerances to wastewaters. When Fitzgibbon et al. (1998) studied the effect of molasses spent wash concentration on fungal growth rates they observed *G. candidum* and *P. chrysosporium* growth rates increased in the presence of increasing concentrations of molasses spent wash up to 50%, while the growth of *M. sterilia* and *T. versicolor* was inhibited at concentrations above 5%.

3.4. Cyclic voltammetry and differential pulse voltammetry

The results for the CV and DPV are briefly summarised in Table 3. Cyclic voltammetry and DPV data for phenolic compounds were very helpful in that they gave a good indication as to the probability that the compounds would be susceptible to oxidation by laccase. The wine lees had a total phenolic compounds concentration that was three times greater than the highest distillery wastewater value, and had a far greater concentration of electro-active compounds.

Of the distillery wastewaters, S1 contained the greatest amount of electro-active compounds, followed by S2 and some electro-active compound was evident in brandy distillery sample B1. The second brandy distillation wastewater (B2) contained electro-active species, but at very low concentrations. Wastewater DL displayed no distinct peaks in the cyclic voltammogram other than a small, concentration dependent peak at 0.8 mV vs. Ag/AgCl. This indicated that even though DL contained the greatest concentration of total phenolic compounds of the distillery wastewater samples, it had no electro-active compounds and the phenolic compounds present would have the lowest tendency to be substrates for laccase oxidation.

3.5. Total phenolic compounds

Table 4 shows that the concentrations of phenolic compounds in the samples ranged from 31 to 566 mg/l. The colours of the wastewaters ranged from light yellow (B2), dark yellow (B1), brown (DL), red (S1 and S2) to dark purple (L). These observations indicated that the phenolic compounds' concentrations and composition varied widely, in agreement with results obtained using CV and DPV, which also indicated substantial variations in phenolic compounds concentration and electro-activities.

The results from the enzymatic treatment showed rapid degradation of phenolics (28–52% removal) within three hours for the samples that were red to purple. The degradation of phenolic compounds in the yellow to brown samples only ranged from 14% to 26%, indicating that the phenolic compounds were not as susceptible to non-mediator assisted enzymatic degradation. After 48 h there was appreciably greater relative phenolic degradation in the yellow to brown samples (26–45%), indicating that slower degradation kinetics were involved. Increased degradation was evident in the red to purple samples over the first 3 h (ranging from 40% to 61%) and the majority of the degradation had occurred relatively quickly. This was attributable to the compounds in the red to purple samples containing more electro-active compounds (i.e. with lower oxidation potentials). The wine lees wastewater (L) had an

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Table 4
Initial concentrations of total phenolic compounds and the percentages of enzymatic and fungal removal

Sample and concentration	Original [TP] ± SD[3]	Laccase treatment		Fungal treatment	
		3 h ± SD[2]	48 h ± SD[2]	48 h ± SD[3]	12 days ± SD[3]
B1 100	280 ± 1.8	26 ± 0.5	33 ± 0.3	46 ± 3.4	79 ± 2.2
B2 100	31 ± 4.2	14 ± 0.8	45 ± 4.9	33 ± 5.4	48 ± 7.8
S1 100	315 ± 0.8	46 ± 0.8	52 ± 0.5	82 ± 4.1	83 ± 0.3
S1 75	249 ± 0.3	42 ± 3.3	52 ± 0.6	80 ± 3.5	81 ± 4.4
S1 50	168 ± 0.7	44 ± 1.6	49 ± 1.6	86 ± 1.5	81 ± 1.5
S2 100	301 ± 2.9	33 ± 1.1	41 ± 0.3	47 ± 2.4	61 ± 1.3
S2 75	228 ± 0.9	33 ± 0.6	41 ± 0.4	54 ± 2.7	72 ± 4.2
S2 50	149 ± 0.9	28 ± 1.5	40 ± 2.4	67 ± 5.2	76 ± 1.5
DL 40	223 ± 1.9	16 ± 1.4	26 ± 1.8	30 ± 2.4	60 ± 2.5
DL 30	152 ± 1.5	16 ± 0.8	28 ± 1.6	48 ± 2.6	71 ± 1.7
DL 20	112 ± 6.6	20 ± 0.4	27 ± 2.4	77 ± 4.3	71 ± 1.1
L 40	566 ± 1.7	51 ± 0.6	61 ± 0.5	68 ± 3.1	87 ± 1.6
L 30	436 ± 2.2	52 ± 0.8	60 ± 0.3	70 ± 1.1	78 ± 1.0
L 20	292 ± 4.4	47 ± 0.7	54 ± 0.5	71 ± 2.1	81 ± 1.0

SD^n: Standard deviation$^{\text{number of replicates}}$.
[TP]: total phenolic compounds concentration in mg/l.

extremely electro-active component while both of the brandy distillation samples and the distilled lees had very low electro-active compounds concentrations. Although the distilled lees sample (DL) had a relatively high concentration of total phenolic compounds, there were no peaks in the voltammograms (summarised in Table 2), indicating that the phenolic compounds had E^0 values greater than 800 mV, making direct laccase-oxidation unlikely.

The fungal treatment displayed much greater removal of phenolic compounds than the enzymatic treatment alone. The fungal treatment achieved greater removal of phenolic compounds after 48 h for every wastewater sample and dilution (Table 4). The removal efficiencies of the phenolic compounds ranged from 33% to 77% for the yellow to brown samples and 47% to 86% for the red to purple samples. After a 12 day digestion the degradation efficiencies had increased to between 48% and 79% for the yellow to brown samples and between 61% and 87% for the red to purple samples.

Two of the samples (L and DL) were inhibitory to fungal growth at full strength and had to be substantially diluted to facilitate treatment. The wine lees (L) had an extremely high total phenolic compounds concentration and was known to contain ethanol, both of which can inhibit microbial metabolism. Sample DL had the highest total phenolics and COD concentrations of the distillery wastewaters and may have contained a number of inhibitory compounds. The lowest results for removal of phenolic compounds were obtained with the distilled lees (DL) wastewater. Although DL contained a high concentration of phenolic compounds, they were found to be very inactive electrochemically. Significantly greater DL degradation was obtained by the fungal culture compared to the enzyme. Only 26–28% of the phenolic compounds were removed by the enzyme, whereas 60–71% were removed by the complete fungal system, indicating that fungal metabolism allowed for a much greater removal of phenolic compounds. These compounds were probably utilized as a carbon source and broken down in a manner other than direct catalysis by laccase.

The concentration of phenolic compounds in the control flasks in the complete cultures increased from zero to 16 mg/l during the fungal treatment, showing that the fungus had synthesised or released phenolic compounds. These phenolic compounds may have acted as mediators and allowed for greater removal of the original phenolic compounds present in the wastewater than the treatment incorporating only the enzyme. Alternatively mediators may be synthesised from the breakdown of more complex phenolic compounds. Mediator synthesis by *P. cinnabarinus* has been demonstrated with lignin degradation (Eggert et al., 1996). Often compounds that are found to be good mediators are also recalcitrant and as, or even more, toxic than the compounds they are intended to remediate. Camerero et al. (2005) evaluated a number of less toxic mediator compounds and found phenolic aldehydes, ketones, acids and esters related to the three lignin units to be among the best mediators with dye degradation. Syringaldehyde and acetosyringone were particularly promising due to high dye degradation efficiencies, lower cost and lower toxicity compared to other mediators tested.

3.6. Colour

The colour of distillery wastewater is another factor that may prevent a wastewater's untreated released into the environment as the darkening of the receiving waters is detrimental to photosynthetic microorganisms. The greatest colour removal by the laccase treatment was a 12% decrease in colour in the S1 wastewater (Table 5) at 100% strength. Although this laccase is known to display optimal kinetic activities from pH 3.0 to 4.5 (Galhaup et al., 2002a), which allowed for significant degradation of phenolic compounds,

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Table 5
Colour removal efficiency (%) of enzymatic and fungal treatment (negative values indicate increases in colour)

Sample and dilution	Laccase treatment		Fungal treatment	
	3 h ± SD[2]	48 h ± SD[2]	48 h ± SD[3]	12 days ± SD[3]
B1 100	0.5 ± 0.6	−0.4 ± 0.4	80 ± 4.2	86 ± 2.0
B2 100	−1.1 ± 1.6	3.3 ± 4.7	81 ± 3.1	70 ± 6.5
S1 100	0.2 ± 0.7	12.0 ± 0.7	77 ± 0.5	80 ± 5.5
S1 75	−1.4 ± 1.2	7.9 ± 0.3	81 ± 2.1	79 ± 0.6
S1 50	1.5 ± 1.3	1.6 ± 1.5	87 ± 1.0	83 ± 0.0
S2 100	0.0 ± 0.8	3.6 ± 0.8	79 ± 1.3	76 ± 3.9
S2 75	−0.2 ± 1.5	3.3 ± 0.9	78 ± 2.6	84 ± 2.3
S2 50	0.0 ± 1.4	5.0 ± 1.9	73 ± 3.8	74 ± 2.7
DL 40	0.7 ± 1.4	1.7 ± 0.3	67 ± 1.2	77 ± 1.9
DL 30	1.1 ± 0.9	4.0 ± 1.4	71 ± 1.6	82 ± 2.2
DL 20	−0.8 ± 0.0	5.3 ± 1.9	88 ± 4.7	76 ± 5.6
L 40	−61.1 ± 1.7	−152.4 ± 4.4	54 ± 0.8	82 ± 1.6
L 30	−146.0 ± 1.4	−143.6 ± 1.2	61 ± 5.4	88 ± 1.0
L 20	−159.8 ± 4.7	−143.1 ± 3.3	74 ± 1.1	90 ± 0.9

SD^n: Standard deviation$^{\text{number of replicates}}$.

Table 6
Maximum laccase activity, days on which the maximum activities occurred and the relative fold increase compared to the control

Sample	HLA[a] (U/l)	Day of HLA[a]	Fold increase
Control	260	12	1
B1 100	8997	9	34.6
B2 100	2847	12	11.0
S1 100	3354	10	12.9
S1 75	2266	7	8.7
S1 50	1491	7	5.7
S2 100	180	3	0.7
S2 75	171	3	0.7
S2 50	1833	8	6.2
DL 40	125	2	0.5
DL 30	2043	7	7.9
DL 20	1650	7	6.3
L 40	2929	10	11.3
L 30	1535	3	5.9
L 20	2133	3	8.2

[a] Highest laccase activity.

there was no significant decrease in colour in the majority of the samples during laccase treatment. The colour of wine lees wastewater increased significantly, even though laccase phenolic degradation efficiencies were the highest in these samples (54–61%).

The results for colour removal by laccase treatment were poor compared to the complete fungal system. After 48 h the colour of all samples treated by the fungal cultures had decreased by 54–88% and after 12 days the colour had been lowered by 74–90%. The wine lees, which was the most colour rich of the wastewaters, had its colour increased by 61% by the action of laccase, but decreased by up to 90% by the complete culture. This indicated that even though laccase present in the complete culture would have led to a colour increase, the fungal mycelia removed the resulting intermediate degradation compounds further, such that the colour was vastly improved from an initial deep purple to a final bright yellow. This corroborated results obtained by Tsioulpas et al. (2002), who observed that several *Pleurotus* spp. were able to grow in OMW without any addition of nutrients and any pretreatment other than media sterilisation. The black colour of OMW became yellow/brown and brighter as the strains grew and 69–76% of the phenolic compounds were removed, with lowest phenolic compound concentrations reached after 12–15 days.

González et al. (2000) tested the ability of a *Trametes* sp. for colour and COD removal in a culture medium supplemented with 20% (v/v) vinasse, which stimulated laccase synthesis 35-fold. The increase in laccase activity corresponded to better colour removal (73% colour removal after seven days), demonstrating the importance of this enzyme with regards to colour removal. The advantage of using a laccase-producing fungus used in the present study is evident when comparing it to fungi that do not produce the enzyme. Three *Penicillium* spp. and *Aspergillus niger* were studied for treating beet molasses alcoholic fermentation wastewater and only removed 40% of the colour (Jiménez et al., 2003).

In the flask cultures containing only laccase there was formation of dark, insoluble phenolic aggregates due to the polymerisation of some of the phenolic compounds. These were clearly visible as suspended solids causing a dark haze and were removed by centrifugation. In the fungal cultures the dark haze was removed after two to three days by the fungal mycelial, resulting in a clear light yellow wastewater with only mycelial pellets evident as suspended solids.

3.7. Laccase synthesis

With all wastewater samples the only adjustments made were to the pH or to the wastewater concentration. The highest laccase synthesis obtained was with brandy distillery wastewater B1 (Table 6). At 8997 U/l, laccase activity was nearly three times higher than the second highest activity (obtained in full strength S1). The combination of availability of a carbon source in the COD with the potential inducers (the presence of copper and phenolic compounds) led to an extremely high synthesis of laccase. Galhaup et al. (2002b) found glucose as a carbon source at a concentration of 40 g/l to be optimal for laccase synthesis using *T. pubescens* MB 89 in a synthetic media. The other brandy distillation sample (B2) also had a relatively high copper concentration, but its potentially poor carbon source (due to the low COD) and very low total phenolics concentration may have hampered the synthesis of laccase. The production of laccase for all wastewaters and concentrations in which an activity of greater than 1800 U/l was observed is shown in Fig. 2. Wastewater S1 had a moderate copper concentration (5.63 mg/l), a fairly high total phenolic compounds con-

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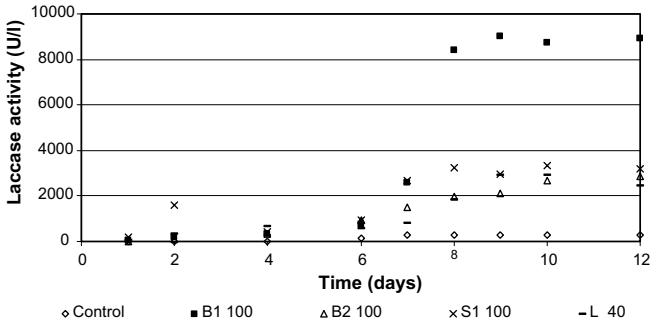

Fig. 2. Laccase synthesis for wastewaters and concentrations in which *T. pubescens* synthesised laccase at concentrations greater than 2900 U/l and the control.

centration (320 mg/l) and an intermediate COD (19.9 g/l). This was enough to allow for the second highest laccase synthesis when tested at full strength. Interestingly, wastewater S1 was the only sample that produced an appreciable concentration of laccase constitutively (associated with growth and primary metabolism). This is evident from the high laccase activity displayed on day two in Fig. 2.

Wine lees had a very high total phenolics concentration (even at a concentration of 40%) and contained ethanol, both of which are known to increase laccase synthesis. Unfortunately, the lack of a sufficient concentration of an easily utilisable carbon source coupled to the lowest copper concentration (aggravated further by dilution) may have hampered very high synthesis, but even so an activity just below 3000 U/l was observed in the 40% concentration. Very low activities were obtained with wastewater DL at or above 40% and for wastewater S2 at or above 75%. Wastewater DL had a very high COD concentration for a wine-related distillery waste, even after the tartaric acid had been extracted and the ethanol removed by distillation. The low laccase activities obtained were attributable to fungal growth inhibition, as shown by the poor COD removal efficiencies over the twelve days of digestion, which negated laccase synthesis. Laccase synthesis could not be correlated to electroactivities of the phenolic compounds present in the wastewaters. The weaker brandy distillery wastewater (B2) displayed high laccase synthesis even though it had an extremely low concentration of phenolic compounds. This indicated the importance of copper, even if only at low concentrations, with respect to allowing for greater laccase synthesis. The highest laccase activities recorded in 20% and 30% concentrations of the wine lees occurred on the third day after inoculation (Table 6). The wine lees had the greatest concentration of electroactive compounds and it was possible that these compounds may have stimulated the early synthesis of laccase.

These results compare favourably to other agricultural waste residues used to produce laccase, as one distillery wastewater produced 8997 U/l with only its pH modified. Osma et al. (2007b) cultured the same fungal strain, but using solid–substrate fermentation of banana skins, and managed to obtain laccase synthesis of 1570 U/l. In other work Osma et al. (2007a) obtained laccase activities of 400 U/l using static flask cultures of *Trametes pubescens* MB89. Mandarin peels served as a carbon source and laccase synthesis was optimised with the addition of soy oil.

4. Conclusions

Trametes pubescens MB 89 greatly improved the quality of all six wastewaters tested, although two wastewaters (wine less and the distilled wine lees) had to be diluted to below 50% to allow for bioremediation by the submerged fungal culture. The fungal culture displayed much better properties than the enzyme alone in removing both the total phenolic compounds and colour. Fungal treatment resulted in a decrease in the COD of up to $83 \pm 2.1\%$. The greatest degradation of phenolic compounds by the fungal culture was $87 \pm 1.6\%$ in contrast to $61 \pm 0.5\%$ by laccase alone. Colour removal of up to $88 \pm 4.7\%$ was attained by the submerged fungal culture, while the highest removal by laccase was only $12 \pm 1.6\%$.

Enzymatic treatment reduced the total phenolic compounds but did little to improve the colour of the wastewaters, and in the case of wine lees significantly increased the colour. The wine lees dilutions contained the highest levels of phenolic and electro-active compounds and displayed the most dramatic results with enzymatic treatment. Although laccase treatment resulted in total phenolics decreases of up to $61 \pm 0.5\%$, the colour was increased by up to $160 \pm 5\%$, indicating that the new compounds formed by laccase were more colour rich than the parent compounds. The complete fungal system was found to be superior to enzymatic treatment alone.

Laccase synthesis greater than 1500 U/l was obtained in all wastewaters. A concentration of 8997 U/l was obtained in the rebate wastewater having an initial COD of 29.5 g/l, total phenolic compounds concentration of 280 mg/l and a copper concentration of 21.9 mg/l. Only its pH was modified (from 3.75 to 4.50).

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