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Enhanced production of ligninolytic enzymes and decolorization of molasses distillery wastewater by fungi under solid state fermentation

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Abstract Selected isolates of fungi were grown on wheat straw and corncob in the presence of different moistening agents such as water, molasses, potato dextrose broth and distillery effluent. All the fungal isolates responded differently with respect to growth and ligninolytic enzyme production. Fungal growth on different substrates was checked by calculating ergosterol content, which varied widely within a single species when grown on different substrates. The maximum laccase production was obtained for *Aspergillus flavus* TERI DB9 grown on wheat straw with molasses. For manganese peroxidase, highest production was in *Aspergillus niger* TERI DB20 grown on corncob with effluent. Among the two isolates positive for lignin peroxidase, the highest production was in *Fusarium verticillioides* ITCC 6140. This immobilized fungal biomass was then used for decolorization of effluent from a cane molasses based distillery. Maximum decolorization (86.33%) was achieved in *Pleurotus ostreatus* (Florida) Eger EM 1303 immobilized on corncob with molasses in a period of 28 days.

Keywords Decolorization · Distillery effluent · Ergosterol · Fungal biomass · Ligninolytic enzymes · Solid state fermentation

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Introduction

White-rot fungi constitute a diverse ecophysiological group comprising mostly of basidiomycetous and litter-decomposing fungi. These fungi exhibit extensive bioremediation activities that are mainly based upon their capabilities to produce one or more extracellular lignin-modifying enzymes (Wesenberg et al. 2003). Lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and laccase are the three major lignin-degrading enzymes with great potential in industrial applications (D'Souza et al. 2006). Production of these enzymes from white-rot fungi has been well documented. However, in recent years, there are several reports of these ligninolytic enzymes being produced from other fungi like *Phylosticta*, *Aspergillus*, *Fusarium* and *Penicillium* (Sahoo and Gupta 2005; Shah et al. 2005; Kumari et al. 2002). Recently, laccase, lignin peroxidase, xylanase, endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase production by *Aspergillus* sp. on agricultural waste of banana under solid

state fermentation (SSF) condition was reported by Shah et al. (2005). Extracellular MnP production under alkaline conditions has been reported in *Aspergillus terreus* (Kanayama et al. 2002). Laccase production in *Fusarium proliferatum* cultures, using wheat bran as a natural lignin–carbon source and benzyl alcohol as laccase inducer has been reported by Fernaud et al. (2006). Edible and medicinal mushroom *Pleurotus* are well known for their ability to produce extracellular ligninolytic enzymes: laccase (Lac), two peroxidases: Mn dependent peroxidase (MnP), versatile peroxidase (VP) and aryl-alcohol oxidase (AAO) (Muñoz et al. 1997). Recently production of laccase and MnP by *Pleurotus eryngii*, *P. ostreatus* and *P. pulmonarius* both under conditions of submerged fermentation (SF) and SSF was reported (Stajić et al. 2006).

Phanerochaete chrysosporium in submerged mode has been used for decolorization of olive oil mill wastewater (Kissi et al. 2001). Earlier, a peroxidase from *Geotrichum candidum* has been used for decolorization of dyes (Kim and Shoda 1999). In recent years, SSF has gained ground as a mean of enhancing enzyme production in fungi. Immobilization is considered as a natural state for fungi, since in nature most fungi tend to attach firmly on surfaces (Pandey et al. 2001). Thus, artificially immobilized microorganisms tend to produce extracellularly secondary metabolites. Wheat straw is the most common among all the various substrates that have been employed for this purpose (Pickard et al. 1999; Aikat and Bhattacharyya 2000; Valášková and Baldrian 2006). Recently sago hampas was used (Vikineswary et al. 2006) for laccase production by *Pycnoporus sanguineus* in SSF. Corncob has also been used by several researchers as a SSF substrate for enhanced enzyme production (Couto and Rättö 1998; Cabaleiro et al. 2002; Oliveira et al. 2006).

These lignin-degrading fungal enzymes lack substrate specificity thus making them capable of degrading a wide range of xenobiotics including industrial colored wastewaters such as dyes and distilleries (Novotný et al. 2001; Pant and Adholeya 2006a). Distilleries are among the most polluting industries generating large amount of wastewater known as molasses spent wash (MSW). In India, there are 319 distilleries at present, producing 3.25×10^9 l of alcohol and generating 40.4×10^{10} l of wastewater annually (Uppal 2004), thus making this problem particularly acute. This dark brown colored effluent, when discharged into water bodies, defiles the natural ecosystem (FitzGibbon et al. 1998). The conventionally treated effluent has dark brown color, strong objectionable odor and contains COD in the range of 25–30,000 mg l^{-1} thereby making it imperative to explore new microorganisms and methods for its effective treatment.

The objective of this study was to investigate the production of ligninolytic enzymes by fungi isolated from distillery effluent and effluent contaminated soils. An attempt was made to enhance the production of these ligninolytic enzymes by using two agricultural residues wheat straw and corncob powder as substrate. The effect of different extractants viz. water and buffer in enzyme recovery is evaluated. The subsequent use of this immobilized fungal biomass in molasses distillery wastewater decolorization has been reported. Further, the role of different moistening agents on fungal growth and enzyme yield is also discussed.

Materials and methods

Microorganisms

The soil samples and effluent after primary treatment was collected from effluent dumping site of Associated Alcohols and Breweries Limited, Barwaha, Madhya Pradesh, a distillery in central India. Sampling was done three times in a year during different seasons in order to have maximum diversity of microorganisms. Throughout sampling, the soil pH was in between 8.5 and 9.5. The soil was serially diluted 10-fold in 0.85% saline, and diluted sample (0.1 ml) was spread on the potato dextrose agar (PDA) plate. The plates were incubated at 25°C for 4 days. The microbial colonies (fungi) that appeared on the PDA plates were isolated and purified. These were characterized at Indian Type Culture Collection (ITCC) at Indian Agricultural Research Institute (IARI), New Delhi, India based on their morphological

structures such as color, diameter of the mycelia and microscopic observation of spore formation. One isolate *Pleurotus ostreatus* (Florida) EM 1303 was procured from the Centre for Mycorrhizal Culture Collection (CMCC), TERI, New Delhi, India. All these isolates were maintained and subcultured on PDA media (Hi Media, India) plates at 25°C.

Preparation of SSF substrates and culture conditions

The water holding capacity was estimated to be 100 ml per 50 g of wheat straw and 150 ml per 50 g of corncob powder. The culture bottles were filled with wheat straw and corncob such that 80% of their volume was left as headspace to increase the oxygen transfer inside the lignocellulose complex material. The moisture content for each substrate was adjusted to 75% (v/w). Four different types of moistening agents viz., water, 1% (v/v) molasses, 100% distillery effluent and Potato Dextrose Broth (PDB, a commercial media) were used to check their effect and suitability for enhancing fungal growth and subsequent enzyme production. The bottles were subsequently sealed and autoclaved for 60 min at 121°C and cooled to room temperature prior to inoculation. After sterilization, each flask was surface inoculated with five discs containing culture mycelium and agar, 6 mm in diameter, from agar plate cultures (4–5 day old) of the fungi on PDA medium. Three replications were prepared for each treatment, and an uninoculated flask served as control. All the flasks were incubated at 25°C in the dark.

Enzyme extraction from SSF substrates

This was done according to an existing method (Makkar et al. 2001) and its slight modifications. Here, water and buffer were used for enzyme extraction to check the difference in extracted enzyme yield. Briefly, mycelium growing on the substrates was taken along with the substrate after 10 days and extracted with 1:2.5 (w/v) 50 mM triethanolamine–maleic buffer, pH 6.0, with continuous stirring. Another set was extracted using 1:2.5 (w/v) water. The extraction step was repeated five times. Extracts obtained were finally pooled and filtered through a glass filter. Resulting filtrate was centrifuged at 10,000 rpm for 25 min. Coloring materials mainly consisting of polyphenolic compounds in the supernatant (Forrester et al. 1990) were removed by addition of cross-linked form polyvinylpyrrolidone (PVP) at a final concentration of 7.7% (w/v) (Loomis 1969). The extracts thus obtained were filtered through a glass filter and processed for the second PVP treatment followed by centrifugation (10,000 rpm, 25 min) to remove fine PVP particles. The treatment with PVP removed the interferences in the enzymatic assays caused either due to the phenolics or polymeric aromatic compounds (Fenice et al. 2003). The decolorized extracts were filtered through Millipore filters (pore size, 0.22 μm) and finally centrifuged at 16,000 rpm for 60 min. Samples were processed at 4°C in all steps.

Measurement of ergosterol

Ergosterol, a component of fungal cell wall was used as a measure of fungal biomass on various substrates. Ergosterol content in different substrates after SSF was measured by an earlier described method (Martin et al. 1990). About 50 mg of fungal mycelium covered SSF substrate was ground in a microfuge tube using a plastic pestle. About 1 ml of absolute ethanol added and the tube was shaken for 30 s, set in ice for 1 h and then centrifuged for 5 min at 14,000 rpm. The supernatant was collected and pellet was resuspended in 1 ml of absolute ethanol and treated once again as mentioned above. The two supernatants were pooled, filtered using 0.22 μm nitrocellulose filters (Millipore) and the filtrate analyzed for ergosterol on a HPLC (Agilent 1100 series, Agilent technologies, Deutschland). Ergosterol was detected, separated and quantified using C-18 column, 150 × 4.6 mm (SS Wakosil, HG, SGE). The samples were eluted with 97:3 methanol/water (v/v) with a flow rate of 0.5 ml min^{-1} and monitored at 282 nm using variable wavelength UV detector. About 50 μl of filtrate was injected into the HPLC system. Peak surface area was measured and compared to the data obtained with standard of known

ergosterol concentration, which was injected before and after each series of sample using ergosterol procured from Sigma chemicals (79% pure).

Enzyme activity determination

Lignin peroxide (LiP), (EC 1.11.1.14) activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde at 37°C as indicated by an increase in A_{310} (Tien and Krik 1988). The reaction mixture (2.5 ml) contained 500 μl enzyme extract, 500 μl H_2O_2 (2 mmol l^{-1}), 500 μl veratryl alcohol solution (10 mmol l^{-1}) and 1.0 ml sodium tartrate buffer pH 3.0 (10 mmol l^{-1}). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 μmol of substrate per minute. Manganese Peroxidase (MnP), (EC 1.11.1.13) activity was measured with phenol red as the substrate at A_{610} (Kuwahara et al. 1984). Reaction mixture contained 500 μl enzyme extract, 100 μl phenol red solution (1.0 g l^{-1}), 100 μl sodium lactate pH 4.5 (250 mmol l^{-1}), 200 μl bovine serum albumin solution (0.5%), 50 μl manganese sulfate (2 mmol l^{-1}) and 50 μl H_2O_2 (2 mmol l^{-1}) in sodium succinate buffer pH 4.5 (20 mmol l^{-1}). Activity is expressed as increase in A_{610} per minute per milliliter. One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 μmol of substrate per minute. Laccase (EC 1.10.3.2) activity is determined by the oxidation of 2, 2′-azino-bis (3-ethylthiazoline-6-sulfonate), i.e., ABTS at 37°C (Buswell and Odier 1987). The reaction mixture (total volume 1 ml) contained 600 μl enzyme extract, 300 μl sodium acetate buffer pH 5.0 (0.1 M) and 100 μl ABTS solution (1 mM). Oxidation was followed via the increase in absorbance at 420 nm. One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 mmol of ABTS per minute.

Effluent decolorization

The different SSF substrates covered with fungal mat were evenly homogenized under sterile conditions and equal amounts (5 g of immobilized substrate) were used to inoculate 100 ml of distillery effluent in a 500 ml Erlenmeyer flask as described previously (Pant and Adholeya 2006b). Distillery effluent used for decolorization studies had undergone hydroponic treatment to reduce high nitrogen content (data not shown). In this process effluent was treated using root zone system of *Phragmites kharka* and *Vetiveria zizanoides*. Physico-chemical characteristics of this effluent are given in Table 1. Controls with no fungal inoculations were also used to account for decolorization due to natural microbial action. In order to rule out effluent decolorization due to adsorption, same amount of heat killed fungal biomass was used for inoculation. Incubation was carried out without agitation for 28 days at 25°C. The flasks were prepared in triplicates for all the treatments. The absorbance of effluent was measured at 475 nm. Decolorization was calculated according the formula given (Itoh 2005): Decolorization (%) = [(initial absorbance–observed absorbance)/initial absorbance] × 100. In order to check the decolorization due to substrates alone, non-sterile blank controls were taken to mimic the actual effluent from the industries where effluent was inoculated with wheat straw and corncob without any fungal growth on them.

Table 1 Physico-chemical characteristics of distillery effluent

Parameter	Anaerobically treated effluent (released in field)
Electrical conductivity (mS cm^{-1})	33.16
pH	8.20
BOD_5 (ppm)	5,000
COD (ppm)	25,000
Total Kjeldahl Nitrogen (%)	3.50
Sodium (ppm)	500
Potassium (ppm)	2,500
Manganese (ppm)	259.44
Magnesium (ppm)	98.00
Zinc (ppm)	272.97
Copper (ppm)	395.51
Total dissolved solids (ppm)	21,256
Total sugar (%)	2.80
Reducing sugar (%)	0.23

Pant et al. (2006)

Statistical analysis

In all the experiments described in this study triplicates were set up for each parameter tested. Completely randomized design was used and sampling was random. The means of three replicate values for all data in the experiments obtained were tested in a One-way analysis of variance using the Costat software (CoHort, Berkeley).

Results and discussion

Identification of isolated fungi

The search for newer strains of fungi producing ligninolytic enzymes and their use in decolorization of industrial wastewaters especially from distilleries is a continuous process. Apart from white rot fungi, which have been exploited so far, there are fungi from other groups as well which can be used for this purpose. A number of fungi were isolated from both the effluent as well as soils contaminated with this effluent. Based on their morphology, the isolates with bioremediation potential were identified as *Aspergillus flavus* TERI DB9, *Fusarium verticillioides* ITCC 6140, *Aspergillus niger* TERI DB18 and *A. niger* TERI DB20.

Ergosterol based biomass estimation

Ergosterol is the membrane component of most fungi and ergosterol levels are commonly used to estimate fungal biomass on various substrates (Charcosset and Chauvet 2001). For instance, in a recent study, ergosterol of basidiomycete *Ganoderma lucidum* was used to study its growth after its solid state fermentation on cornmeal (Han et al. 2005). In our study, all isolates exhibited different growth response on different SSF substrates as evidenced by varying amount of ergosterol produced (Table 2). Even within the same substrate, the effect of different moistening agents was quite pronounced. In *A. flavus* TERI DB9, maximum ergosterol was detected when grown on wheat straw with PDB, which was significantly higher than corncob with PDB. The minimum ergosterol in case of *A. flavus* TERI DB9 was when grown on

Table 2 Ergosterol content ($\mu g\ g^{-1}$) in fungal isolates grown on wheat straw and corncob wetted with different moistening agents

Isolate	Water		Molasses		Effluent		PDB	
	Wheat straw	Corncob	Wheat straw	Corncob	Wheat straw	Corncob	Wheat straw	Corncob
A. flavus TERI DB9	$32.86^{c} \pm 2.98$	$72.95^{b} \pm 9.97$	$40.83^{c} \pm 2.65$	$82.47^{b} \pm 5.05$	$68.40^{b} \pm 1.22$	$39.46^{c} \pm 1.45$	$114.81^{a} \pm 3.20$	$63.46^{b} \pm 0.50$
LSD (0.01)	18.18							
F. verticillioides ITCC 6140	$76.53^{bc} \pm 3.94$	$141.72^{a} \pm 3.37$	$60.93^{c} \pm 1.75$	$128.201^{a} \pm 25.90$	$74.22^{bc} \pm 5.53$	$110.36^{ab} \pm 7.16$	$108.54^{ab} \pm 4.50$	$105.4^{abc} \pm 3.62$
LSD (0.01)	41.76							
A. niger TERI DB18	$56.85^{c} \pm 2.50$	$96.77^{b} \pm 2.17$	$101.87^{b} \pm 3.31$	$35.00^{d} \pm 2.13$	$232.83^{a} \pm 4.29$	$45.77^{cd} \pm 3.39$	$113.47^{b} \pm 8.33$	$63.89^{c} \pm 4.11$
LSD (0.01)	17.45							
A. niger TERI DB20	$69.44^{f} \pm 4.57$	$73.40^{ef} \pm 3.32$	$85.49^{de} \pm 2.59$	$41.61^{g} \pm 2.19$	$92.17^{cd} \pm 1.75$	$98.82^{c} \pm 3.40$	$138.27^{a} \pm 2.41$	$115.61^{b} \pm 2.17$
LSD (0.01)	12.11							
P. ostreatus (Florida) EM 1303	$222.64^{b} \pm 27.62$	$84.43^{c} \pm 11.45$	$196.80^{b} \pm 21.26$	$464.61^{a} \pm 12.77$	$176.34^{b} \pm 35.37$	$235.51^{b} \pm 0.18$	$201.93^{b} \pm 5.42$	$238.53^{b} \pm 13.12$
LSD (0.01)	79.48							

Mean ± standard error. Means within a row followed by the same superscript letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.01$)

wheat straw with water. However, this was not significantly different from its growth on wheat straw with molasses, and corncob with effluent. The maximum ergosterol in *F. verticillioides* ITCC 6140 was observed when grown on corncob powder with water. However, this was not significantly different from the one when it was grown on corncob with molasses or effluent or PDB and wheat straw with PDB. The growth was significantly less when grown on wheat straw with water, molasses or effluent. For *A. niger* TERI DB18, growth was maximum, when grown on wheat straw with distillery effluent as moistening agent which was significantly higher than all other treatments. This was followed by growth on wheat straw with PDB, wheat straw with molasses and corncob with water, which did not differ significantly. The growth was significantly less when it was grown on wheat straw with water, corncob with effluent and corncob with PDB. In *A. niger* TERI DB20, growth in terms of ergosterol was highest on growing it on wheat straw with PDB followed by corncob on PDB and corncob on effluent. All other treatments resulted in significantly reduced growth. *P. ostreatus* (Florida) Eger EM 1303 recorded maximum growth on corncob with molasses which was significantly higher than rest of the treatments. There was no significant difference in growth due to all the other treatments except for corncob with water where growth was significantly less.

SSF based enzyme production

For enzyme production, SSF mode was chosen in this study since it has been reported that production of ligninolytic enzymes is repressed by agitation in submerged liquid culture (Galhaup et al. 2002). Most of the studies so far have been carried out in submerged liquid culture conditions or solid cultures on agar plates, which do not reflect the natural living conditions of these fungi (Boer et al. 2004). Agriculture residues such as wheat straw and corncob powder as a support provides the fungus a similar environment to its natural habitat and offers the possibility of re-using an agricultural waste. Recently, enhanced production of *P. ostreatus* (Florida) EM 1303 on wheat straw amended with distillery effluent was reported by Pant et al. (2006).

Table 3 Screening results for ligninolytic enzyme production (U ml^{-1})

Isolate	Laccase	MnP	LiP
A. flavus TERI DB9	$0.45^{a} \pm 0.01$	$0.51^{b} \pm 0.05$	ND
F. verticillioides ITCC 6140	ND	$0.34^{b} \pm 0.08$	$2.03^{a} \pm 0.35$
A. niger TERI DB18	ND	$1.12^{a} \pm 0.14$	ND
A. niger TERI DB20	ND	$0.81^{ab} \pm 0.76$	$0.89^{b} \pm 0.08$
P. ostreatus (Florida) EM 1303	$0.81^{a} \pm 0.11$	ND	ND

ND—Not detected. Mean ± standard error. Means within a column followed by the same superscript letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.01$)

Among the five selected fungi, only two, *A. flavus* TERI DB9 and *P. ostreatus* (Florida) EM 1303 were found as laccase producers (Table 3). Like ergosterol, enzyme production also followed the similar trend with same isolate producing varying amount of enzyme when grown on different SSF substrates (Table 4). Also, extraction with water and buffer influenced the amount of enzyme detected. In *A. flavus* TERI DB9, maximum laccase production was detected when grown on wheat straw with effluent and extracted with water, which was significantly higher from the one, which was extracted with buffer. All other treatments had no significant difference on laccase production by *A. flavus* TERI DB9. With *P. ostreatus* (Florida) EM 1303, maximum laccase was detected when grown on corncob with molasses and extracted with buffer, which was non-significantly different from when this isolate was grown on corncob with PDB; wheat straw with molasses and extracted with buffer, wheat straw with water and extracted with water and buffer. In general, extraction with water gave significantly reduced yield of enzyme for *P. ostreatus* (Florida) EM 1303 when grown on wheat straw with effluent and PDB, and corncob with water and effluent. Earlier, Bucher et al. (2004) reported the production of wood decay enzymes including laccase from tropical

Table 4 Laccase production (U ml^{-1}) by fungal isolates in SSF with different substrates

Isolate	Wheat straw								Corncob							
	Water		Molasses		Effluent		PDB		Water		Molasses		Effluent		PDB	
	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer
A. flavus TERI DB9	0.097^{abc} ± 0.013	0.068^{bc} ± 0.013	0.194^{abc} ± 0.01	0.098^{abc} ± 0.006	0.347^{a} ± 0.048	0.048^{e} ± 0.003	0.095^{abc} ± 0.018	0.109^{ab} ± 0.108	0.247^{abc} ± 0.013	0.241^{abc} ± 0.013	0.164^{abc} ± 0.006	0.563^{abc} ± 0.425	0.276^{abc} ± 0.006	0.154^{abc} ± 0.009	0.079^{bc} ± 0.015	0.231^{abc} ± 0.023
LSD (0.01)	0.226															
P. ostreatus (Florida) EM 1303	0.238^{abcd} ± 0.007	0.265^{abc} ± 0.015	0.225^{bcd} ± 0.014	0.255^{abc} ± 0.051	0.11^{efg} ± 0.013	0.12^{efg} ± 0.023	0.057^{g} ± 0.008	0.265^{abc} ± 0.036	0.177^{cde} ± 0.012	0.296^{ab} ± 0.012	0.106^{efg} ± 0.004	0.323^{a} ± 0.019	0.073^{fg} ± 0.006	0.151^{def} ± 0.023	0.221^{bcd} ± 0.01	0.288^{ab} ± 0.032
LSD (0.01)	0.083															

Mean ± standard error. Means within a row followed by different superscript letters are significantly different according to Duncan's Multiple Range Test ($P \leq 0.01$)

freshwater fungi. Recently, laccase production by two strains of *Aspergillus* has been reported (Souza et al. 2005), which were further used for treatment of delignification effluent from a nitrocellulose industry.

Four isolates viz., *A. flavus* TERI DB9, *F. verticillioides* ITCC 6140, *A. niger* TERI DB18 and *A. niger* TERI DB20 were found positive for MnP (Table 5). In *A. flavus* TERI DB9, maximum MnP was detected when grown on corncob powder with molasses and extracted with water, which was not significantly different when extracted with buffer. Minimum yield was observed when *A. flavus* TERI DB9 was grown on wheat straw with water and extraction was also with water. For *F. verticillioides* ITCC 6140, maximum MnP was detected when grown on wheat straw with water, and extraction with water, which again was not significantly different from its extraction with buffer. Growth on wheat straw with effluent and extraction with water and buffer also did not vary significantly when grown on wheat straw with molasses. However, rest of the treatments resulted in significantly reduced MnP yield with minimum in case of growth on corncob with molasses and extracted with buffer. MnP in *A. niger* TERI DB18 was found highest when grown on corncob with molasses and extracted with buffer. The minimum was found when *A. niger* TERI DB18 was grown on wheat straw with water and extracted with buffer. Here, in many treatments such as wheat straw with PDB and corncob with water, non-significant difference was observed in yield irrespective of the extractant used. Similar trend was observed when grown on corncob with effluent and PDB. For *A. niger* TERI DB20, maximum yield was found in corncob with effluent, and extraction with water. Again this was not significantly different when extracted with buffer. Among other treatments, there was no significant difference in MnP yield when *A. niger* TERI DB20 was grown on wheat straw with water and PDB, corncob with water, molasses and PDB irrespective of the extractant. Earlier MnP production by *A. terreus* has been reported (Kanayama et al. 2002). Besides this, the expression of *P. chrysosporium* MnP in *A. niger* has also been reported (Conesa et al. 2000). Low levels of MnP in certain isolates of *Fusarium*

Table 5 MnP production (U ml^{-1}) by fungal isolates in SSF with different substrates

Isolate	Wheat straw								Corncob							
	Water		Molasses		Effluent		PDB		Water		Molasses		Effluent		PDB	
	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer
A. flavus TERI DB9	0.254^{d} ± 0.042	0.927^{cd} ± 0.054	2.818^{a} ± 0.195	0.29^{bc} ± 0.047	0.65^{cde} ± 0.072	0.419^{bc} ± 0.033	1.129^{cd} ± 0.13	0.321^{bc} ± 0.027	0.8^{cd} ± 0.054	0.448^{cd} ± 0.059	3.909^{a} ± 0.445	3.834^{a} ± 0.332	0.351^{d} ± 0.054	0.291^{d} ± 0.045	0.643^{cd} ± 0.033	0.568^{cd} ± 0.042
LSD (0.01)	0.635															
F. verticillioides ITCC 6140	0.568^{ef} ± 0.04	0.845^{def} ± 0.066	2.392^{a} ± 0.137	2.22^{a} ± 0.072	2.19^{a} ± 0.369	1.936^{ab} ± 0.221	1.271^{cd} ± 0.061	1.121^{cde} ± 0.098	1.532^{bc} ± 0.061	1.226^{cd} ± 0.06	0.605^{ef} ± 0.149	0.501^{f} ± 0.095	0.807^{def} ± 0.104	0.717^{def} ± 0.045	0.859^{def} ± 0.095	0.845^{def} ± 0.04
LSD (0.01)	0.521															
A. niger TERI DB18	0.284^{ef} ± 0.052	0.217^{f} ± 0.027	0.762^{cd} ± 0.127	0.717^{cde} ± 0.085	1.749^{a} ± 0.263	1.33^{b} ± 0.166	0.389^{def} ± 0.049	0.351^{def} ± 0.054	0.433^{def} ± 0.045	0.404^{def} ± 0.047	1.016^{bc} ± 0.13	1.951^{a} ± 0.093	0.688^{cde} + 0.094	0.673^{cde} ± 0.047	0.71^{cde} ± 0.042	0.673^{cde} ± 0.034
LSD (0.01)	0.401															
A. niger TERI DB20	0.419^{e} ± 0.033	0.404^{e} ± 0.047	3.677^{abc} ± 0.557	0.934^{e} ± 0.122	3.296^{bc} ± 0.059	2.688^{cd} ± 0.157	1.779^{de} ± 0.084	1.166^{e} ± 0.085	0.957^{e} ± 0.176	0.852^{e} ± 0.113	0.389^{e} ± 0.02	0.359^{e} ± 0.026	4.918^{a} ± 1.088	4.223^{ab} ± 0.371	0.957^{e} ± 0.251	0.874^{e} ± 0.25
LSD (0.01)	1.322															

Mean ± standard error. Means within a row followed by different superscript letters are significantly different according to Duncan's Multiple Range Test ($P \leq 0.01$)

Table 6 LiP production (U ml^{-1}) by fungal isolates in SSF with different substrates

Isolate	Wheat straw						Corncob					
	Molasses		Effluent		PDB		Molasses		Effluent		PDB	
	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer
F. verticillioides ITCC 6140	9.29^{bc} ± 0.68	8.96^{cd} ± 0.73	34.14^{a} ± 3.523	6.64^{cde} ± 2.758	3.943^{de} ± 0.629	5.332^{de} ± 0.613	2.12^{de} ± 0.06	4.96^{de} ± 0.28	3.5^{de} ± 0.26	1.944^{e} ± 0.287	4.409^{de} ± 0.398	1.792^{cde} ± 0.010	6.631^{de} ± 1.269	2.742^{de} ± 0.317	17.9^{b} ± 0.94	2.491^{de} ± 0.116
LSD (0.01)	5.685															
A. niger TERI DB20	2.39d^{e} ± 0.12	3.39^{cd} ± 0.42	4.543^{c} ± 0.173	1.613^{e} ± 0.117	23.86^{a} ± 0.327	9.444^{b} ± 0.987	3.72^{cd} ± 0.11	1.37^{e} ± 0.38	4.23^{c} ± 0.11	3.224^{cd} ± 0.203	4.014^{cd} ± 0.476	4.803^{c} ± 0.276	2.509^{de} ± 0.059	1.55^{e} ± 0.282	3.78^{cd} ± 0.46	3.199^{cd} ± 0.259
LSD (0.01)	1.433															

Mean ± standard error. Means within a row followed by different superscript letters are significantly different according to Duncan's Multiple Range Test ($P \leq 0.01$)

solani have also been observed (Saparrat et al. 2000).

Two isolates namely, *F. verticillioides* ITCC 6140 and *A. niger* TERI DB20 were positive for LiP (Table 6). In *F. verticillioides* ITCC 6140, growth on wheat straw with molasses and water extraction recorded maximum LiP yield followed by corncob with PDB and water extraction. Rest of the treatments gave significantly reduced yield with minimum when grown on corncob with water and extraction with buffer. In case of *A. niger* TERI DB20, maximum LiP production was found when grown on wheat straw with effluent, and extraction with water, which was significantly higher from the one extracted with buffer. The minimum yield in this case, was for growth on corncob powder with effluent and buffer extraction. LiP has been reported from *Aspergillus* sp., isolated from a mangrove area whose best activity was in coir pith substrate at 3% concentration (Ahammed and Prema 2002). Recently LiP production by *Penicillium decumbens* was reported (Yang et al. 2005). Although the enzymatic system related with decolorization of melanoidins is yet to be completely understood, it seems greatly connected with fungal ligninolytic mechanisms. One of the enzymatic studies regarding melanoidin decolorization was reported by Miyata et al. (1998). Color removal of synthetic melanoidin by *C. hirsutus* involved the participation of peroxidases (MnP and MIP) and the extracellular H_2O_2 produced by glucose–oxidase, without disregard of a partial participation of fungal laccase. These authors used *C. hirsutus* pellets to decolorize a melanoidin containing medium. It was elucidated that extracellular H_2O_2 and two extracellular peroxidases, a manganese–independent peroxidase (MIP) and MnP were involved in decolorization activity.

Distillery effluent decolorization

Maximum decolorization of distillery effluent achieved was 86.33% in case of *P. ostreatus* (Florida) EM 1303 grown on corncob with molasses after 28 days of incubation, followed by 74.67% decolorization by *A. flavus* TERI DB9

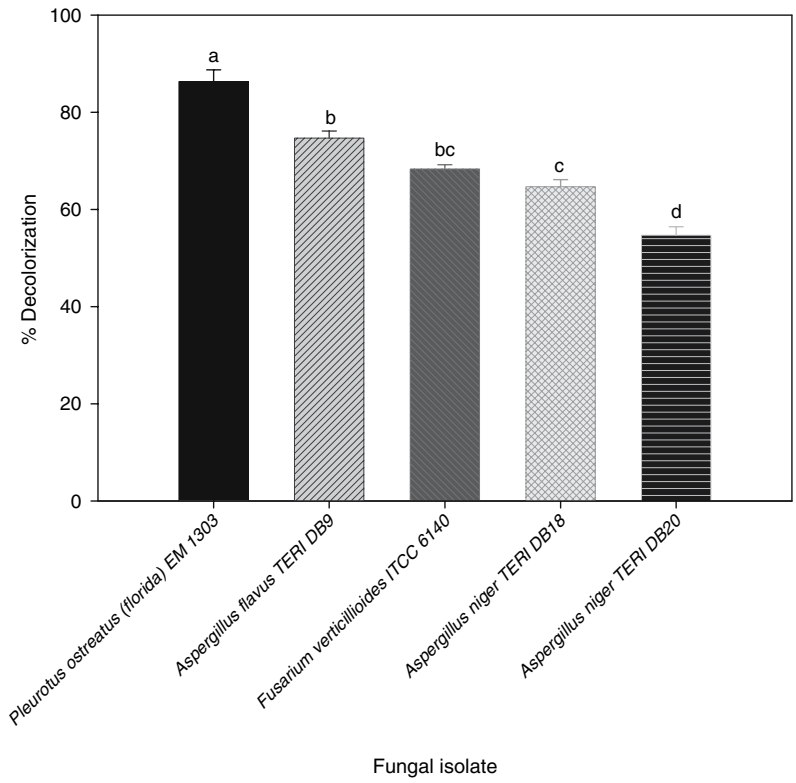

Fig. 1 Effluent decolorization by fungal isolates. Letters above the histogram bars represents analysis of variance (ANOVA). Bars with different letters indicate means with significant difference. Bars represent mean of three replicates at $P \leq 0.01$

grown on wheat straw with effluent, 68.33% by *F. verticillioides* ITCC 6140 grown on wheat straw with molasses, 64.67% by *A. niger* TERI DB18 grown on wheat straw with effluent and 54.67% by *A. niger* TERI DB20 grown on wheat straw with effluent (Fig. 1). Besides this, there was a reduction in pH, COD and BOD of the effluent by all the fungi (Fig. 2). Prior to this, Kahraman and Yesilada (2003) reported molasses decolorization in solid state cultivation by fungi *Coriolus versicolor*, *Funalia trogii*, *P. chrysosporium* and *Pleurotus pulmonarius* with cotton stalks being used as additional source of carbon. *C. versicolor* decolorized 48% of 30% diluted vinasse without any additional carbon source which increased to 71% on addition of cotton stalks. Very recently 100% decolorization of 10% spent wash by a marine fungal isolate was reported whose laccase production was increased several folds in the presence of phenolic and non-phenolic inducers (D'souza et al. 2006). Miranda et al. (1996) reported 69% color removal when $MgSO_4$, KH_2PO_4, NH_4NO_3 and a carbon source was added to wastewater from alcohol fermentation involving beet molasses.

Color removal by adsorption is ruled out in this case, since no decolorization of effluent was observed in wheat straw and corncob without the fungal inoculum. In the heat-killed immobilized fungus treatment, no decolorization was noticed throughout the experiment. Using *Flavodon flavus*, a white-rot basidiomycete fungus isolated from a marine habitat 73% decolorization of 10% diluted molasses spent wash (MSW) was achieved in 7 days (Raghukumar et al. 2004). However, in this case decolorization was achieved in 50% diluted effluent. It was proposed that hydrogen peroxide produced as a result of enzyme activity might act as a bleaching agent. Also since the production of H_2O_2 in several fungi depend on substrates used and other culture conditions, several workers have attempted to correlate the production of ligninolytic enzymes in white-rot fungi and the rates of decolorization. It has been shown that it is possible to stimulate the yield of laccase activity of *T. versicolor* by using several

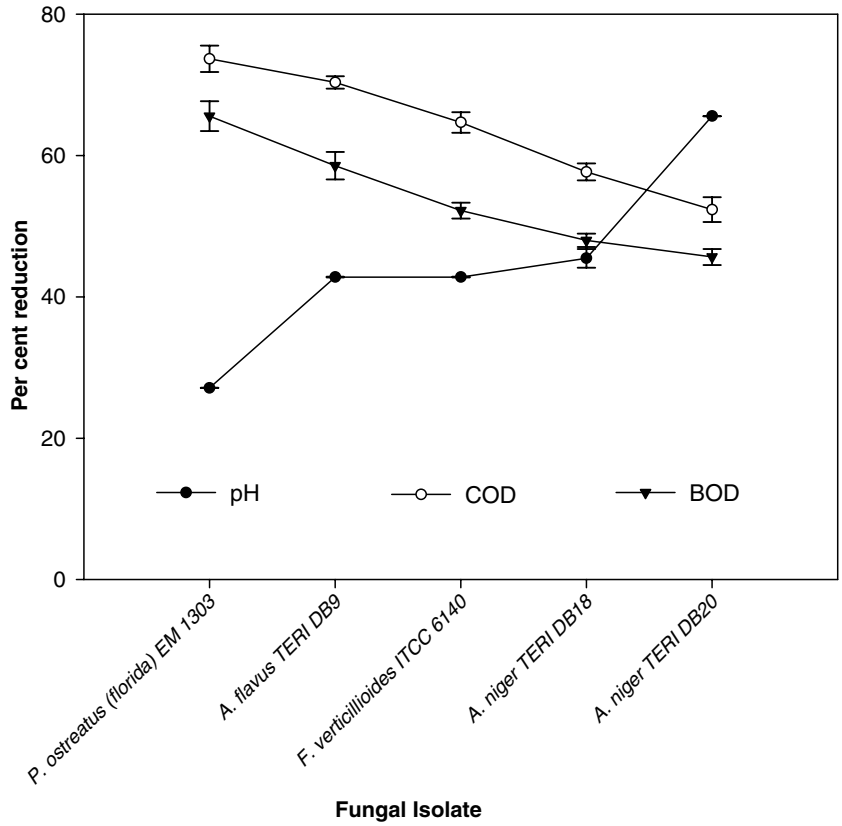

Fig. 2 Per cent change in pH, COD and BOD over control by fungal isolates

agricultural wastes (Lorenzo et al. 2002); however, the decolorizing capacity of the extracellular liquid was not found proportionately increased.

Conclusions

This is the first report of decolorization of highly recalcitrant distillery effluent using indigenously isolated fungi at a higher concentration (50% v/v). The previous studies carried out for the decolorization of distillery effluent can be linked by the fact that almost all of them used effluent at lower dilutions ranging from 6.5% (Kumar et al. 1998) to 20% (Gonzalez et al. 2000). To the best of our knowledge, this is also the first report for extracellular MnP and LiP activities of *F. verticillioides*. The results of this study clearly show the suitability of wheat straw and corncob powder moistened with different liquids such as molasses and distillery effluent as a support medium for enhanced production of enzymes by fungi. These agro-residues would be an effective supplement to support the decolorization process because of their low cost and easy availability in most tropical and subtropical developing countries. Besides, their usage is environmentally safe and no extra carbon and/or nitrogen source in the media is required, when this material is used for immobilization process to fungus. After immobilization, the fungi could be employed in a reactor such as a rotating biological contractor (RBC). This may be a practically economic and easily employable technique for treating large volumes of distillery wastewaters.

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References

Ahammed S, Prema P (2002) Influence of media nutrients on synthesis of lignin peroxidase from *Aspergillus* sp. Appl Biochem Biotechnol 103:327–336

Aikat K, Bhattacharyya BC (2000) Protease extraction in solid state fermentation of wheat bran by a local strain of *Rhizopus oryzae* and growth studies by the soft gel technique. Process Biochem 35:907–914

Boer CG, Obici L, deSouza CGM, Peralta RM (2004) Decolorization of synthetic dyes by solid state cultures of *Lentinula (Lentinus) edodes* producing manganese peroxidases as the main ligninolytic enzyme. Bioresour Technol 94:107–112

Bucher VVC, Pointing SB, Hyde KD, Reddy CA (2004) Production of wood decay enzymes, loss of mass, and lignin solubilization in wood by diverse tropical freshwater fungi. Microb Ecol 48:331–337

Buswell JA, Odier E (1987) Lignin biodegradation. CRC Rev Biotechnol 6:1–60

Cabaleiro DR, Rodríguez-Couto S, Sanromán A, Longo MA (2002) Comparison between the protease production ability of ligninolytic fungi cultivated in solid state media. Process Biochem 37:1017–1023

Charcosset JY, Chauvet E (2001) Effect of culture conditions on ergosterol as an indicator of biomass in the aquatic hyphomycetes. Appl Environ Microbiol 67:2051–2055

Conesa ACA, Hondel VD, Punt PJ (2000) Studies on the production of fungal peroxidases in *Aspergillus niger*. Appl Environ Microbiol 66:3016–3023

Couto SR, Rättö M (1998) Effect of veratryl alcohol and manganese (IV) oxide on ligninolytic activity in semi solid cultures of *Phanerochaete chrysosporium*. Biodegradation 9:143–150

D'souza DT, Tiwari R, Sah AK, Raghukumar C (2006) Enhanced production of Laccase by a marine fungus during treatment of colored effluents and synthetic dyes. Enzyme Microb Technol 38:504–511

Fenice M, Sermanni GG, Federici F, D'Annibale A (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. J Biotechnol 100:77–85

Fernaud JRH, A Marina A, González K, Vázquez J, Falcón MA (2006) Production, partial characterization and mass spectrometric studies of the extracellular laccase activity from *Fusarium proliferatum*. Appl Microbiol Biotechnol. 70:212–221

FitzGibbon F, Singh D, McMullan G, Marchant R (1998) The effect of phenolics acids and molasses spent wash concentration on distillery wastewater remediation by fungi. Process Biochem 33:799–803

Forrester IT, Grabski AC, Mishra C, Kelly BD, Strickland WN, Leatham GF, Burgess RR (1990) Characteristics and N-terminal amino acid sequence of a manganese peroxidase purified from *Lentinus edodes* cultures grown on a commercial wood substrate. Appl Microbiol Biotechnol 33:359–365

Galhaup C, Wagner H, Hinterstoisser B, Haltricha D (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme Microb Technol 30:529–536

Gonzalez T, Terron MC, Yague S, Zapico E, Galletti GC, Gonzalez AE (2000) Pyrolysis/gas chromatography/mass spectrometry monitoring of fungal biotreated distillery wastewater using *Trametes* sp. I 62 (CECT 20197). Rapid Commun Mass Spectrom 14:1417–1424

Han JR, An CH, Yuan JM (2005) Solid-state fermentation of cornmeal with the basidiomycete *Ganoderma lucidum* for degrading starch and upgrading nutritional value. J Appl Microbiol 99:910–915

Itoh K (2005) Decolorization and degradation of methylene blue by *Arthrobacter globiformis*. Bull Environ Contam Toxicol 75:1131–1136

Kahraman S, Yesilada O (2003) Decolorization and bioremediation of molasses wastewater by white-rot fungi in a semi-solid state condition. Folia Microbiol 48:525–528

Kanayama N, Suzuki T, Kawai K (2002) Purification and characterization of an alkaline manganese peroxidase from *Aspergillus terreus* LD-1. J Biosci Biochem 93:405–410

Kim SJ, Shoda M (1999) Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec 1 involved in decolorization of dyes. Appl Environ Microbiol 65:1029–1035

Kissi M, Mountadarb M, Assobhei O, Gargiulo E, Palmieri G, Giardina P, Sannia G (2001) Roles of two white-rot basidiomycete fungi in decolorisation and detoxification of olive mill waste water. Appl Microbiol Biotechnol 57:221–226

Kumar V, Wati L, Nigam P, Banat IM, Yadav BS, Singh D, Marchant R (1998) Decolorization and biodegradation of anaerobically digested sugarcane molasses spent wash effluent from biomethanation plants by white-rot fungi. Process Biochem 33:83–88

Kumari M, Yadav RS, Yadav KD (2002) Secretion of ligninperoxidase by *Penicillium citrinum*, *Fusarium oxysporum* and *Aspergillus terreus*. Ind J Exp Biol 40:802–806

Kuwahara M, Glenn JK, Morgan MA, Gold MH (1984) Separation and Characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett 169:247–250

Loomis WD (1969) Removal of phenolic compounds during the isolation of plant enzymes. Methods Enzymol 13:555–563

Lorenzo MD, Moldes D, Rodriguez CS, Sanroman A (2002) Improving laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. Bioresour Technol 82:109–113

Makkar RS, Tsuneda A, Tokuyasu K, Mori Y (2001) *Lentinula edodes* produces a multicomponent protein complex containing manganese (II)-dependent peroxidase, laccase and L-glucosidase. FEMS Microbiol Lett 200:175–179

Martin F, Delaruelle C, Hilbert JL (1990) An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. Mycol Res 94:1059–1064

Miranda PM, Benito GG, Cristobal NS, Nieto CH (1996) Color elimination from molasses wastewater by *Aspergillus niger*. Bioresour Technol 57:229–235

Miyata N, Iwahori K, Fujita M (1998) Manganese-independent and -dependent decolourization of melanoidin by extracellular hydrogen peroxide and

peroxidases from *Coriolus hirsutus* pellets. J Ferment Bioeng 85:550–553

Muñoz C, Guillen F, Martínez TA, Martínez JM (1997). Induction and characterization of laccase in the ligninolytic fungus *Pleurotus eryngii*. Curr Microbiol 34:1–5

Novotný C, Rawal B, Bhatt M, Patel M, Sašek V, Molitoris HP (2001). Capacity of *Irpex lacteus* and *Pleurotus ostreatus* for decolorization of chemically different dyes. J Biotechnol 89:113–122

Oliveira LA, Porto ALF, Tambourgi EB (2006) Production of xylanase and protease by *Penicillium janthinellum* CRC 87M-115 from different agricultural wastes. Bioresour Technol 97:862–867

Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P (2001) Solid-state fermentation in biotechnology. fundamentals and applications. History and development of solid-state fermentation. P-3. Asiatech Publishers, New Delhi

Pant D, Adholeya A (2006a) Biological approaches for treatment of distillery wastewater: a review, Bioresour Technol doi:10.1016/j.biortech.2006.09.027 (in press)

Pant D, Adholeya A (2006b) Isolation and screening of potential fungi for decolourization of distillery wastewaters. In: Mendez-Vilas A (eds.) Modern multidisciplinary applied microbiology exploiting microbes and their interactions. Wiley-VCH, Weinheim, pp 95–102

Pant D, Reddy UG, Adholeya A (2006) Cultivation of oyster mushrooms on wheat straw and bagasse substrate amended with distillery effluent. World J Microbiol Biotechnol 22:267–275

Pickard MA, Vandertol H, Roman R, Duhalt V (1999) High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium. Can J Microbiol 45:627–631

Raghukumar C, Mohandass C, Kamat S, Shailaja MS (2004) Simultaneous detoxification and decolorization of molasses spent wash by the immobilized white-rot fungus *Flavadon flavus* isolated from a marine habitat. Enzyme Microb Technol 35:197–202

Sahoo DK, Gupta R (2005) Evaluation of ligninolytic microorganisms for efficient decolorization of a small pulp and paper mill effluent. Process Biochem 40:1573–1578

Saparrat MCN, Martínez MJ, Tournier HA, Cabello MN, Arambarri AM (2000) Production of ligninolytic enzymes by *Fusarium solani* strains isolated from different substrata. World J Microbiol Biotechnol 16:799–803

Shah MP, Reddy GV, Banerjee R, Babu PR, Kothari IL (2005) Microbial degradation of banana waste under solid state bioprocessing using two lignocellulolytic fungi (*Phylosticta* spp. MPS-001 and *Aspergillus* spp. MPS-002). Process Biochem 40:445–451

Souza JVB, da Silva ES, da Silva FT, Paiva TCB (2005) Fungal treatment of a delignification effluent from a nitrocellulose industry. Bioresour Technol 96:1936–1942

Stajić M, Persky L, Friesem D, Hadar Y, Wasser SP, Nevo E, Vukojević J (2006) Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species. Enzyme Microb Technol 38:65–73

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol 6:238–249

Uppal J (2004) Water utilization and effluent treatment in the indian alcohol industry—an overview. In: Liquid assets, proceedings of Indo-EU workshop on promoting efficient water use in agro-based industries, TERI, New Delhi, 15–16 January 2004, pp 13–19. TERI Press, New Delhi, India

Valášková V, Baldrian P (2006) Estimation of bound and free fractions of lignocellulose-degrading enzymes of wood-rotting fungi *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus*. Res Microbiol 157:119–124

Vikineswary S, Abdullah N, Renuvathani M, Sekaran M, Pandey A, Jones EBG (2006) Productivity of laccase in solid substrate fermentation of selected agroresidues by *Pycnoporus sanguineus*. Bioresour Technol 97:171–177

Wesenberg D, Kyriakides I, Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187

Yang JS, Yuan HL, Wang HX, Chen WX (2005) Purification and characterization of lignin peroxidases from *Penicillium decumbens* P6. World J Microbiol Biotechnol 21:435–440