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Decolourization of pulp mill wastewater using thermotolerant white rot fungi

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ABSTRACT: Twenty isolates of white rot fungi were collected from several provinces in Thailand. Eight isolates could be cultured to test for thermotolerance and to screen for the presence of ligninolytic enzymes using *Phanerochaete chrysosporium* as a reference. Of the eight isolates, only three species *Daedaleopsis* sp., *Schizophyllum commune* PT, and *S. commune* SL were able to grow above 40 °C. All three species exhibited 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)-oxidizing activity on ABTS agar plates. Only *P. chrysosporium* could oxidize manganese on $MnCl_2$ agar plates. Both *P. chrysosporium* and *Daedaleopsis* sp. were able to decolourize wastewater on wastewater agar plates. All fungal cell suspensions tested decolourized wastewater No. 1 (pH 8.07, chemical oxygen demand (COD) 4347 mg/l) from the pulping process and wastewater No. 2 (pH 6.94, COD 4000 mg/l) from the pulping process combined with that from the paper recycling process. *Daedaleopsis* sp. and *P. chrysosporium* exhibited the highest ability to decolourize wastewater No. 1 (52%) and No. 2 (86%), respectively. Laccase activities were detected in the decolourized effluents and all fungi tested reduced the COD by 59–71% (No. 1) and 66–83% (No. 2).

KEYWORDS: wastewater treatment, bioremediation, ligninolytic fungi

INTRODUCTION

The pulp and paper industry typically generates large quantities of wastewater whose correct treatment prior to discharge to the environment represents a significant environmental and economic problem. One significant problem is the persistent dark brown colour in the released effluent from wastewater treatment facilities of which the major contributors are lignin and its derivatives, such as chlorolignin, discharged from the pulp bleaching process[1,2]. These compounds are normally recalcitrant and thus problematic. For several decades attempts have been made to remove the dark colour from the effluents. Whereas, chemical oxidation or precipitation methods are tedious and provide an additional environmental load, biological methods for colour removal are particularly attractive and have been investigated using several groups of microorganisms. Biological methods are of particular interest because they can also reduce the chemical and biological oxygen demands (COD and BOD)[2], which are also significant problems in pulp wastewater, and so reduce holding times in aeration and sedimentation ponds prior to water discharge into the environment. Among the potential biological agents, white rot fungi have been receiving attention due to their efficiency and presence of diverse ligninolytic enzyme systems involving lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). These fungi have been reported as having promising capabilities of decolourizing wastewater from a wide range of industries including those producing olive oil, alcohol, textile, and pulp and paper[1]. In the case of the pulp and paper industries, *Phanerochaete flavidoalba*[3], *Coriolus versicolor*[4], *Aspergillus niger*[5], and *Schizophyllum commune*[6] have been shown to be able to remove the residual recalcitrant colour efficiently from paper and pulp mill effluents.

Thermophilic and thermotolerant fungi are of potential importance to the biotechnology industry since they can be applied to a wider range of bioprocesses than mesophilic fungi and hyperthermophilic archae[7]. Thailand is a tropical country that possesses a diverse array of soil and climatic habitats and a high biodiversity of plants (and thus lignin and lignocellulose substrates) and microbes. In many locations the temperature can exceed 40 °C during the summer months. White rot fungi thriving in such conditions are therefore ideal candidates for isolation and testing for thermotolerant organisms with a practical use.

MATERIALS AND METHODS

Collection and isolation of white rot fungi

Twenty fruiting bodies of white rot fungi were collected from Maewang District, Chiangmai, Pau District, Nan, Kao Kor District, Petchaboon, Wangnamkeaw District, Nakorn Ratchasima, Muang District, Sakaew, Makkasan and Pathumwan Districts, Bangkok, Muang District, Pang-Nga, Hatyai District, Songkhla, and Muang District, Pattani. They were identified based on the morphology of fruiting body, hypha, and spores[8,9]. Pieces of fruiting bodies were aseptically placed onto potato dextrose agar (PDA) plates (Difco) for the isolation of fungal mycelia and were subcultured until pure mycelia were obtained. *Phanerochaete chrysosporium* obtained from the Plant Biomass Utilization Research Unit, Chulalongkorn University, was also included in this study as a relative standard and positive control. Pure mycelia of all fungal isolates were maintained at 4 °C in distilled water.

Thermotolerance test

All the isolated white rot fungi and *P. chrysosporium* were cultured on PDA plates and incubated at 30, 35, 40, 45, 50, 55, and 60 °C. The mycelial growth, measured as colony diameter, was monitored daily. All experiments were performed in triplicate.

Detection of lignin degrading enzyme activities

The ability of the white rot fungi to produce ligninolytic enzymes was tested on agar plates with either ABTS (PDA containing 250 mg/l 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid), Wako) or $MnCl_2$ (PDA containing 0.1 g/l of $MnCl_2 \cdot 4\,H_2O$, Sigma) media. All fungal isolates were precultured on PDA plates at 35 °C for 7 days. One mycelium plug (1 cm in diameter) was placed onto each ABTS or $MnCl_2$ agar plate. The inoculated plates were incubated at 35 °C. A dark green (on ABTS medium) or dark brown (on $MnCl_2$ medium) zone around the fungal colonies was considered a positive result. All plates were observed daily and the experiments were performed in triplicate.

Determination of the properties of pulp and paper mill wastewater

Wastewater from the pulping process (wastewater No. 1) and wastewater from the pulping process combined with that from the paper recycling processes (wastewater No. 2), were collected from the wastewater treatment facility of the Siam Kraft Co. Ltd. pulp and paper factory, Ratchaburi, Thailand. Both samples were centrifuged at 3000g for 20 min to remove heavy sediments, mixed to homogeneity to reduce variations between assays, and measured for pH, absorbance at 475 nm (spectrophotometer, UV 2001, Hitachi), and COD[10].

Decolourization of pulp mill wastewaters on solid media and in liquid cell suspension

The white rot fungi were screened for their abilities to decolourize wastewater using PDA containing 10% (v/v) wastewater No. 1. The plates were incubated at 35 °C for 5 days before the detection of a colour reduction zone was assayed. The experiments were carried out in triplicate.

A conidial spore suspension (10^7 cell/ml) of each fungus was prepared using sterile distilled water containing 0.1% (v/v) Tween 80, and 1 ml of spore suspension was inoculated into 100 ml potato dextrose broth (PDB, Difco) medium and then incubated at room temperature (30 °C) in an orbital shaker (150 rpm) for 3 days. Cell pellets obtained from PDB were filtered through Whatman No. 1 paper and then 4 g wet weight of the cell pellet was aseptically transferred into 20 ml of wastewater medium (No. 1 or No. 2) supplemented with 1% (w/v) glucose (Wako) and 0.12% (w/v) NH_4Cl (Wako) in a 100-ml flask. The cultures were incubated at 35 °C in an orbital shaker (130 rpm), and 1 ml of culture was sampled daily for 4 days. The samples were centrifuged at 7379g for 5 min prior to measurement of pH and absorbance at 475 nm. The ligninolytic activity was assayed in terms of laccase activity using 2,2′-azinobis (3′-ethylbenz thiazoline-6-sulphonate) (ABTS, Wako) as substrate. The assay mixture contained 30 mM ABTS and 0.1 M sodium tartrate buffer (pH 5.0). Oxidation of ABTS was followed by an increase in absorbance at 420 nm (ε = 36 000 $M^{-1}cm^{-1}$). The COD were also determined at day 4 using standard methods[10]. The experiments were performed in triplicate. The amount of colour reduction R was calculated using

$$R = \frac{A - A_0}{A_0}$$

where A_0 and A are, respectively, the absorbances of wastewater and decolourized wastewater at 475 nm.

RESULTS AND DISCUSSION

All fruiting bodies of the white rot fungi collected belonged to the order Polyporales. Pure mycelia were successfully obtained for eight isolates. Based on the morphology, five isolates were identified as *Ganoderma* spp., two were *Schizophyllum commune*,

and one was *Daedaleopsis* sp. Both *Ganoderma* and *S. commune* are common white rot fungi found in various habitats around the world while *Daedaleopsis* sp. has rarely been reported. Some species of *Daedaleopsis* are known to be wood-rotting fungi growing on specific deciduous trees in temperate areas[11], whereas one member of the genus, *Daedaleopsis confragosa*, has been reported as a source of many triterpene derivatives and has some unusual fatty acids in its constituents as well as lignin-degrading enzymes[12].

None of the five *Ganoderma* spp. could grow or survive at or above 40 °C. However, both isolates of *S. commune*, PT (from Pattani province) and SL (from Bangkok province), and the *Daedaleopsis* sp. were found to be thermotolerant. *S. commune* PT and SL grew well at 30–40 °C whereas PT could survive at 45 °C. *Daedaleopsis* sp. was able to grow between 30–45 °C. These fungal isolates were defined as being thermotolerant due to their ability to grow or survive above 40 °C and were selected for further study as detailed below.

The isolates *S. commune* SL, *S. commune* PT, and *Daedaleopsis* sp. together with *P. chrysosporium* as a positive control and reference species were tested for the presence of ligninolytic enzymes on ABTS and $MnCl_2$ agar plates. All fungi grew well on both media but *Daedaleopsis* sp. could form a light-green ring around its colony on ABTS medium within the first day of incubation, whereas the other isolates took longer (Table 1). Assuming all enzymes diffuse equally well and retain similar specific activities under these conditions, these observations suggest that *Daedaleopsis* sp. is a rapid producer of extracellular ABTS-oxidizing enzymes. The positive test for ABTS-oxidizing activities suggests the presence of extracellular LiP and laccase[13]. In contrast, only *P. chrysosporium* gave a positive result on the $MnCl_2$ agar medium, showing brown flecks of MnO_2 (an oxidizing product caused by MnP) after 6 days of incubation and a darker brown colour after 7 and 8 days. The phenomenon of MnP production on the $MnCl_2$ agar medium of *P. chrysosporium* has been observed[13,14] and serves as a useful control thereby supporting the validity of the negative result, under these conditions, for the other three fungal isolates.

Table 1 The ability of thermotolerant white rot fungi to produce and secrete ABTS-oxidizing enzymes on solid PDA plates containing ABTS as indicated from the intensity of colour reactions.

Fungal isolate	Time of incubation (days)							
	1	2	3	4	5	6	7	8
P. chrysosporium	–	–	–	+	++	+++	+++	+++
Daedaleopsis sp.	+	++	+++	+++	+++	+++	+++	+++
S. commune PT	–	–	–	–	–	+	++	++
S. commune SL	–	+	++	++	++	++	++	++

+ = light green, ++ = green, +++ = dark green

Wastewater No. 1 was darker ($A_{475} = 0.775$) than wastewater No. 2 ($A_{475} = 0.225$) and was mildly basic (pH 8.07) whereas wastewater No. 2 was slightly acidic (pH 6.94). The COD values were broadly similar at 4374 and 4000 mg/l, respectively.

When using cell suspension for decolourization, all white rot fungi tested were able to decolourize both wastewaters. However, only *P. chrysosporium* and *Daedaleopsis* sp. were found to decolourize wastewater No. 1 on the solid agar plates whereas *S. commune* SL and PT did not show any detectable colour reduction. Similar results for the wastewater decolourization in a liquid medium by *S. commune* have been reported previously[6]. Perhaps the solid media used in this study were not suitable for detecting the presence of *S. commune* ligninolytic enzymes. Nimchua et al reported that the agar plate assay for detecting the PET-hydrolysing enzyme of *Fusarium* spp. showed no correlation between the quantity of enzyme produced and the clearing zone development[15].

All four fungi decolourized wastewater No. 2 to a greater extent than No. 1 (Fig. 1), suggesting that all the fungi tested might be able to decolourize wastewater more efficiently when there is a lower amount of colour (or lignin) and/or under mildly acidic conditions. For the pulping wastewater No. 1, the degree of colour reduction increased from day 1 to day 4 in all cultures. *Daedaleopsis* sp. showed the highest and fastest ability to decolourize the wastewater No. 1 at 52% on day 4 (Fig. 1). *P. chrysosporium* could also rapidly reduce the colour reaching up to 40% whereas both *S. commune* isolates could remove less than 20% of the colour by day 4 but differed in kinetics with SL initially being faster but slowing down thereafter in contrast to PT.

The pH of inoculated wastewater No. 1 decreased from slightly basic on day 0 to neutral/slightly acidic on day 4 in all four fungal cultures, whereas the COD decreased in all cultures but to slightly different extents (Table 2), with *P. chrysosporium* giving the highest COD reduction.

Although all four fungal isolates released laccase into the media, by far the highest level was detected for *Daedaleopsis* sp. whereas very low levels were detected for *P. chrysosporium*. The production of laccase by *P. chrysosporium* is still unclear. This fungus was routinely reported as a producer for LiP and MnP but not laccase[16]. However, Srinivasan

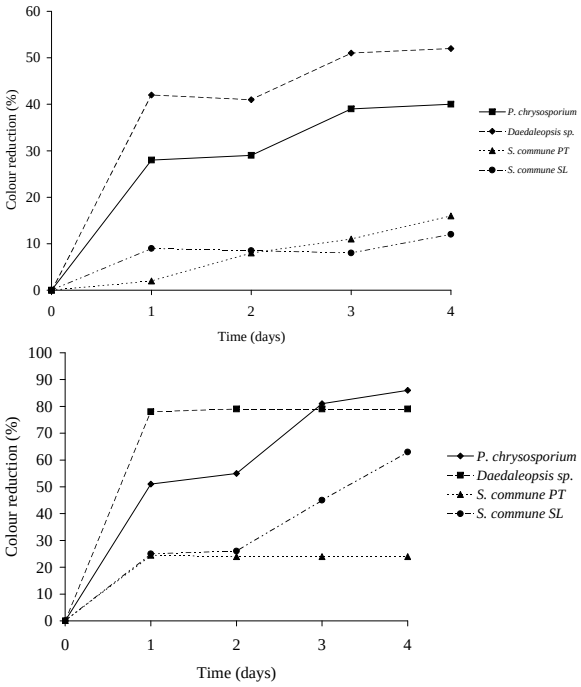

Fig. 1 Time dependence of the colour reduction in wastewaters No. 1 (*top*) and No. 2 (*bottom*) inoculated with white rot fungi.

et al[17] reported that laccase activity could be detected from a culture medium of *P. chrysosporium* BKM-F1767[17].

For wastewater No. 2 the broad trend of colour reduction was similar to that of No. 1. The highest reduction was found in the *Daedaleopsis* sp. and *P. chrysosporium* cultures. However, whereas *Daedaleopsis* sp. could rapidly remove almost 80% of the colour by day 1, compared to only 51% for *P. chrysosporium*, essentially no further colour reduction was detected afterwards. In contrast, *P. chrysosporium*, continued to reduce the colour attaining 86% by day 4. Although, *S. commune* isolates SL and PT showed essentially identical kinetics and rates of colour removal over the first two days attaining 24–27% decolourization, isolate PT then plateaued at this level whereas the rate of decolourization attained by SL increased dramatically reaching a final level of 63% by day 4, in stark contrast to the 18% seen with wastewater No. 1 at day 4.

The pH of all inoculated wastewater No. 2 samples also decreased with time and remained slightly more acidic compared to those of wastewater No. 1. As was the case for wastewater No. 1, *P. chrysosporium* followed by *Daedaleopsis* sp. gave the best COD reduction levels, whereas the highest laccase activity was also detected from the culture of *Daedaleopsis* sp. (Table 2). Overall, *Daedaleopsis* sp. produced the highest level of laccase activity in both wastewaters and was slightly more efficient in colour removal than *P. chrysosporium*, although it reduced COD at a slower rate. It seems likely that *P. chrysosporium* decolourized pulp mill wastewater and reduced COD through different mechanisms using other ligninolytic enzymes such as LiP and/or MnP rather than laccase[16].

The ability to decolourize pulp mill wastewater by these white rot fungi was found to be in agreement with several previous reports. Several strains of white rot fungi have been found to decolourize wood processing wastewater[18] with *P. chrysosporium* being the most common. For instance, Calvo et al showed that *P. chrysosporium* could decolourize wheat straw alkaline-cooking effluents by up to 50% after 7 days in a shake-flask cultivation system[19]. Some white rot strains such as *Ceriopsis subvermispora* could decolourize kraft-bleaching effluent at 90% and also resulted in reduction of COD of up to 45% which correlated well to our recent report[18]. However, higher percentages of COD, BOD, and total solids reduction in pulp mill wastewater have been reported using a reduction-biological technique[20]. In Thailand, thermotolerant white rot fungi, such as *Coriolus versicolor*, *Pycnoporus* spp., and *Lenzites* spp., have been isolated from the cooler northern region and used for

Table 2 COD, pH, and laccase activity of wastewater media at day 4 after inoculation with fungal cell pellets.

Fungal isolate	COD (mg/l)		pH		Lac activity (U/ml)[a]	
Wastewater No.:	1	2	1	2	1	2
Control (no cells added)	4374	4000	8.07	6.94	0	0
P. chrysosporium	1287	669	6.32	5.49	0.0054	0.0022
Daedaleopsis sp.	1535	1072	7.09	6.26	0.0795	0.0473
S. commune PT	1807	1254	6.33	6.44	0.0050	0.0086
S. commune SL	1700	1361	7.08	5.62	0.0157	0.0086

[a] 1 U is the amount of enzyme that oxidizes 1 µmol of ABTS per min

dye decolourization[4]. However, our investigation is the first to describe the use of thermotolerant white rot fungi and the more efficient *Daedaleopsis* sp. for the decolourization of pulp and paper wastewater.

CONCLUSIONS

Three of the isolates of white rot fungi obtained from natural habitats in Thailand were thermotolerant and capable of secreting active ligninolytic enzymes and decolourizing two types of pulp mill wastewater. *Daedaleopsis* sp. was found to be a new fungal isolate that exhibited high decolourization activity on pulp mill wastewaters when used as cell pellet suspension in a shake-flake culture. These thermotolerant fungi are likely to be both more common than currently known and to be important natural resources. In particular, their ability to decolourize wastewater at higher temperatures makes them more suitable for tropical environments.

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