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Decolorization of Textile Dyes by Extracellular Enzymes Produced from *Trametes sanguinea* and *Perenniporia taephropora* Immobilized on Natural Media

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ABSTRACT

The color of textile wastewater is still a main problem in wastewater treatment by biological processes. The colored effluents from textile factories usually exceed effluent standards. Therefore, various innovations were developed to treat textile wastewater for decolorization in the effluents. This research aims to decolorize textile wastewater by immobilizing white rot fungi degradation. At first, the 11 fungal stains were tested to find the decolorized efficiency then the high decolorized efficiency fungal stains were immobilized on four material media, namely water hyacinth stalks, coconut husk, corn cob, and loofah. After that, the immobilized fungi were cultivated in the culture media at 30, 60, and 120 C/N ratios, respectively. The results showed that *Trametes sanguinea* and *Perenniporia tephropora* were two stains with a high decolorized efficiency of 68.8% and 67.5% respectively, and the decolorized efficiency was increased when immobilized on loofahs and fed with 120 C/N ratio medium. In a comparison of two fungal stains, *P. tephropora* was found more suitable for the decolorization of textile wastewater than *T. sanguinea* because *T. sanguinea* could produce red-orange pigments that induced the colored enhancement in wastewater over time. Finally, immobilized *P. tephropora* was cultivated in a 120 C/N ratio medium within a 10 L continuous stirred tank reactor (8 L working volume) to investigate the decolorized efficiency, enzymatic activity, and repeated batch. It was found that three repeated cycles were carried out by reusing the immobilized *P. tephropora* and the highest decolorized efficiency was 63.4%. The enzymatic activity of laccase, manganese peroxidase, and lignin peroxidase was 15.5 U/L, 85.9 U/L, and 0 U/L, respectively

INTRODUCTION

The textile dyeing industry is important to the economic development of Thailand. Currently, there are 159 textile dyeing factories in Thailand (Department of Industrial Works 2022), and they have continuously exported garments that generate income for the country. During the production process, high volumes of water are used in various steps such as de-sizing, scouring, bleaching, and dyeing, while chemicals and dyes are applied to improve the properties of the fabric. As a result, a huge quantity of wastewater is discharged and dark color. Characteristics of these wastewaters are high suspended solids and BOD values (Pollution Control Department 2022) which are caused by organic substances from the dyeing process (starch, fiber, dye, and solvents) and containing heavy metal inorganic compounds (copper, lead, chromium, cobalt and zinc) from dyes, so the effluent has a color and high pH value (The Bureau of Science and Technology Information Service 2022). It is necessary to have decolorization and wastewater treatment processes in accordance with the wastewater standards of the Department of Industrial Works before releasing it into natural water sources. Synthetic organic dyes such as azo dyes and disperse dyes are now widely used in the textile dyeing industry. It was found that about 10-15% of dye components were leaked from the dyeing process into the effluent (Naghizadeh & Nabizadeh 2016).

The active methods for textile wastewater decolorization can be treated in many ways, both physical and chemical treatments, including chemical sedimentation, ozone oxidation, adsorption by activated carbon, and membrane technology (Sachidhanandham & Periyasamy 2021). These methods are costly and leave chemical residues in the environment. Consequently, it's not popular nowadays. Decolorization with the biological process by using white rot fungi is the best choice to be suitable and highly effective for decoloring wastewater from the textile dyeing industry (Zainith et al. 2020). White rot fungi can produce extracellular enzymes such as laccase, lignin peroxidase,

and manganese peroxidase to decompose derivatives of ethanolamine in azo dyes and anthraquinone dyes which can decolorize the industrial wastewater.

In addition, a lot of research studied ligninolytic enzyme production by white rot fungi and its decolorization efficiency. Patel et al. (2014) found that *Pleurotus ostreatus* HP-1 can excellently produce laccase enzyme and is stable at 70°C. Cheng et al. (2007) studied manganese peroxidase produced from *Schizophyllum* sp. F17, which is stable under pH 4-7 at 25°C. Kong et al. (2016) investigated manganese peroxidase from *Echinodontium taxodii* 2538 for lignin degradation, it has the highest enzyme activity at pH 3.5 and 55°C. Lignin peroxidase enzyme was produced in the white rot fungi, *Trametes versicolor* (Manavalan et al. 2015) and *Phanerochaete chrysosporium* (Bilal & Iqbat 2020). Moreover, white rot fungi have also been used in the decolorization treatment of industrial wastewater such as *Daedaleopsis* sp. and *P. chrysosporium* (Prasongsuk et al. 2009), *Phanerochaete chrysosporium*, *Trametes versicolor* (Costa et al. 2017); *Pleurotus ostreatus* (Haider 2019); *Trametes hirsuta* PW17-41 (Tampropaporn et al. 2022), It was found that the efficiency of color removal was in the range of 70-90%.

Therefore, this research aims to select white rot fungi strains that have high efficiency in removing color in real dyeing industrial wastewater by immobilizing cells on supporting media (corn cob, coconut husk, loofah, water hyacinth stalk) coupled with varying C/N ratios in the culture medium inside the continuous stirred tank reactor (CSTR) with a working volume of 8 L to increase filament forming capacity and obtain high decolorized efficiency and enzymes.

MATERIALS AND METHODS

Microorganisms

Nine isolates of white-rot fungi were obtained from the Department of Microbiology, Faculty of Science, Srinakharinwirot University, Thailand. These fungi were *Coriolopsis retropicta* (MK589270), *Ganoderma* sp. (MK589271), *Ganoderma* sp. (MK589274), *Ganoderma* sp. (MK589275), *Microporus* sp. (MK589280), *Microporus* sp. (MK589281), *Trametes elegans* (MK589285), *Trametes sanguinea* (MK589287) and *Pseudolagarobasidium* sp. (MK589289). Two isolates of white rot fungi (*Perenniporia tephropora* (OP358037) and *Pleurotus* sp. (OP358038) were derived from the Department of Agro-Industrial, Food and Environmental Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok. The GenBank accession numbers of internal transcribed spacer sequences were represented in parentheses.

Textile Wastewater and Chemicals

Textile wastewater (containing azo and anthraquinone dyes) was obtained from the aeration tank of the activated sludge process in the textile factory located in Bang Khun Thian District, Bangkok, Thailand. Other chemicals and media were purchased from Sigma-Aldrich (Missouri, USA) Himedia (Mumbai, India), and Ajax Finechem (New South Wales, Australia).

Decolorized Screening by White Rot Fungi

Eleven isolates of white rot fungi were grown on a Potato Dextrose Agar (PDA) plate at 30°C for 7 days. Five plugs, 8 mm in diameter from the growing edge of mycelia in the PDA plate were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of basal medium (pH 5). The basal medium was consisted of 10 $g.L^{-1}$ glucose, 1 $g.L^{-1}$ peptone, 1 $g.L^{-1}$ KH_2PO_4, 0.5 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.05 $g.L^{-1}$ Na_2HPO_4, 0.01 $g.L^{-1}$ $CaCl_2$, 0.01 $g.L^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.001 $g.L^{-1}$ $MnSO_4 \cdot 4H_2O$, 0.001 $g.L^{-1}$ $ZnSO_4 \cdot 4H_2O$, 0.002 $g.L^{-1}$ $CuSO_4 \cdot 5H_2O$ and textile wastewater 100%v/v. Culture media were incubated at 30°C, 100 rpm for 5 days. The experiment was performed in triplicate. Ligninolytic enzyme activities (laccase, MnP and LiP) and color in ADMI color units were assayed.

Fungal Immobilization on Different Media

Four types of media (water hyacinth stalks, coconut husk, corn cob, and loofah) were used for fungal mycelium immobilization. These media were washed with distilled water, dried at 50 °C for 24 hours, and cut into 1 cm^3 cubes. Then, five plugs (8 mm in diameter) of the selected isolate from the growing edge of mycelia were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of Potato Dextrose Broth (PDB) and 5 pieces of media (3 replicates). The culture was shaken at 100 rpm, 30°C for 7 days. The immobilized fungal mycelia on media were taken out, washed with sterile distilled water, and dried at 50°C for 48 hours. The dry weight of mycelia adhered to the four types of media was measured.

Effect of Carbon on Nitrogen Ratios

The effect of carbon-to-nitrogen ratios on wastewater decolorization was observed. Five immobilized fungal mycelia on the selected media were inoculated in 500 mL Erlenmeyer flasks that contained 200 mL modified basal medium (pH 5) which used sucrose and ammonium tartrate as carbon and nitrogen sources, respectively (Tampropaporn et al. 2022, Huang et al. 2020). The C/N ratio in the modified basal medium was varied to 30, 60, and 120. The control treatment was a basal medium used for the decolorized screening. The experiment was tested in triplicate. Culture media were incubated in agitated conditions (100 rpm) at

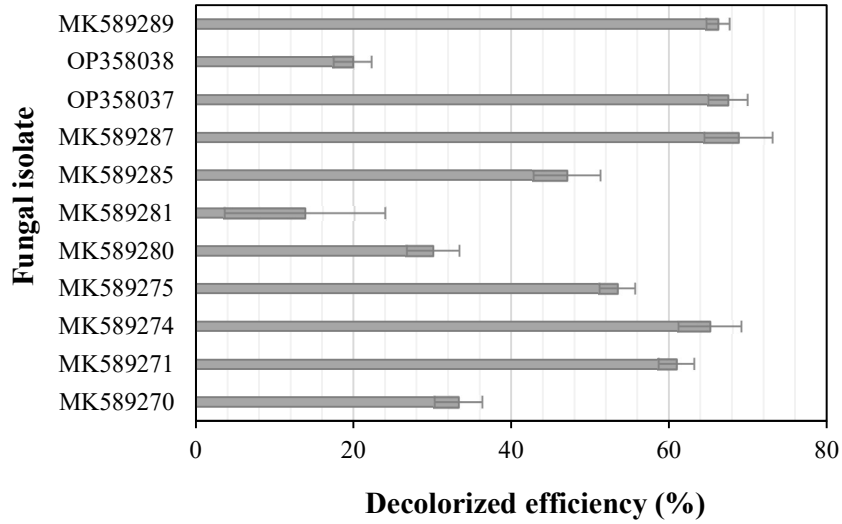

Fig. 1: The highest decolorized efficiency of eleven fungal isolates.

30°C for 6 days. Enzyme activity (laccase, MnP and LiP) and color in ADMI color units were assayed.

Decolorization of Wastewater in Bioreactor

Eighty immobilized fungal mycelia on the selected media were cultured in a 10 L continuous stirred tank reactor (CSTR) containing 8 L modified basal medium with the optimal C/N ratio. The aeration was continuously supplied, and the reactor was kept at 30°C. After the color value dropped below 300 ADMI, the old modified basal medium was replaced by a new modified basal medium, and the replacement was repeated until the decolorization was inefficient. Enzyme activities (laccase, MnP and LiP), the number of replacements, and color in ADMI color units were analyzed.

Analytical Determinations

Ligninolytic enzyme activities (laccase, MnP and LiP) were analyzed following the method of Kietkwanboot et al. (2020). They were expressed as units per liter, in which one unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate per liter per minute. The decolorized efficiency (DE) was calculated from the following equation:

$$DE = (ADMI_0\text{-}ADMI_1)/ADMI_0 \times 100$$

Where, $ADMI_0$ = initial color in ADMI color units

$ADMI_1$ = final color in ADMI color units

RESULTS AND DISCUSSION

Decolorized Screening by White Rot Fungi

Numerous researchers indicate that white rot fungi could break down synthetic dyes because of the production of non-specific extracellular ligninolytic enzymes (Anastasi et al. 2010, Teerapatsakul & Chitadon 2016, Tampropaporn et al. 2022). As a result, eleven isolates of white rot fungi were screened for textile wastewater treatment. *Trametes sanguinea* (MK589287) showed the highest decolorized efficiency (68.8%) followed by *Perenniporia tephropora* (OP358037) (67.5%) (Fig. 1). The highest ligninolytic enzyme activity of 11 isolates white rot fungi was presented in Table 1.

T. sanguinea and *P. tephropora* were reported for application in dye and wastewater decolorization such as anthraquinone dye (Lu et al. 2007), phenolic dye (Ling et al. 2015), acid dye (Younes et al. 2006) and pulp mill effluent (Teerapatsakul & Chitradon 2016). It was not surprising that both isolates showed high decolorization, therefore, they were tested in further experiments.

Table 1: The highest ligninolytic enzyme activity (U/L) of eleven fungal isolates.

Accession number	Ligninolytic enzyme activity (U/L)		
	Laccase	Manganese peroxidase	Lignin peroxidase
MK 589270	120.08	172.97	3.76
MK 589271	10.52	22.12	10.22
MK 589274	52.11	237.21	2.15
MK 589275	8.59	32.00	9.86
MK 589280	0.06	1.46	8.24
MK 589281	0.00	1.88	11.29
MK 589285	0.34	1.70	2.69
MK 589287	273.15	2432.12	2.15
MK 589289	41.35	86.73	1.25
OP 358037	47.16	121.09	11.47
OP 358038	47.10	64.73	1.97

Table 2: Dry weights of *T. sanguinea* and *P. tephropora* on four types of media.

Type of media	Fungal isolate	
	T. sanguinea	P. tephropora
Corn cob	0.000±0.000	0.000±0.000
Water hyacinth stalks	0.000±0.000	0.081±0.010
Coconut husk	0.033±0.013	0.149±0.021
Loofah	0.049±0.007	0.372±0.018

Fungal Immobilization on Different Media

Four different media were observed for fungal immobilization. Dry weights of *T. sanguinea* and *P. tephropora* mycelia on four types of media were displayed in Table 2. The result revealed that loofah was the most suitable media for fungal immobilization while corn cob was so decayed that the fungal mycelium could not adhere to it. The immobilized mycelium dry weight of *P. tephropora* was higher than *T. sanguinea* because the mycelium of *P. tephropora* could grow rapidly and intertwined very tightly. Loofah was the suitable media due to a lot of pores and rough surfaces which the fungal mycelium could insert in the pores and adhere to the rough surface (Tampropaporn et al. 2022). Thus, the fungal mycelium could grow to cover the surface of the loofah.

Effect of Carbon to Nitrogen Ratios

Effects of C/N ratios on decolorized wastewater were examined. The optimal C/N ratio for decolorized efficiency by *T. sanguinea* and *P. tephropora* was 120 at 52.7% and 60.2%, respectively (Fig. 2). For ligninolytic enzyme activity, the highest activity was found in 120 C/N ratio medium and lignin peroxidase was the lowest activity enzyme while manganese peroxidase was the highest activity enzyme (Fig. 3). Anastasi et al. (2010) reported that the decolorized efficiency of most fungi was increased when cultured in high C/N ratio medium because the lack of nitrogen stimulated the production of ligninolytic enzyme such as laccase, manganese peroxidase and lignin peroxidase.

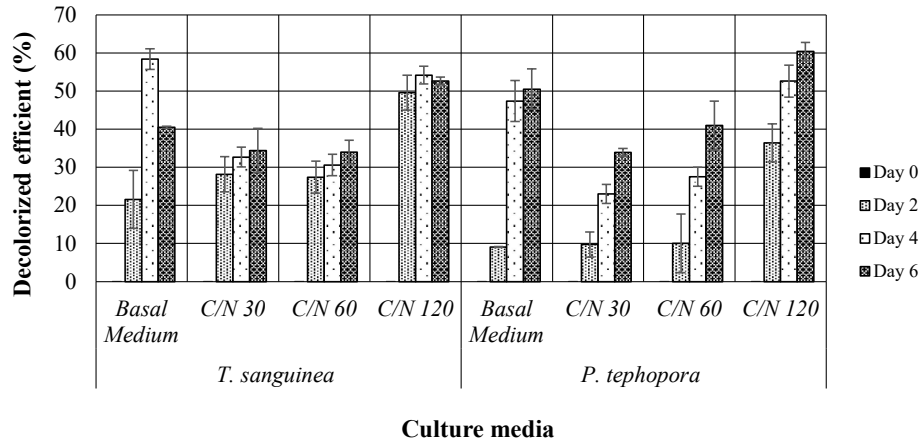

Fig. 2: Decolorized efficiency of *T. sanguinea* and *P. tephropora* in various C/N ratios media.

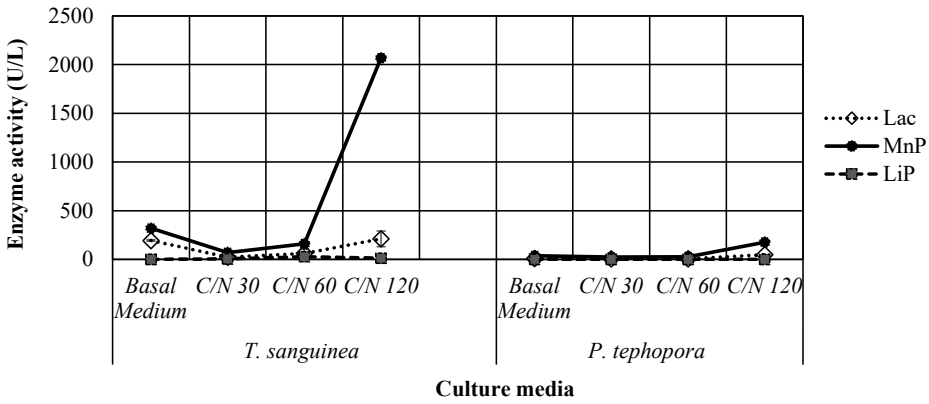

Fig. 3: Ligninolytic enzyme activity of *T. sanguinea* and *P. tephropora* in various C/N ratios media.

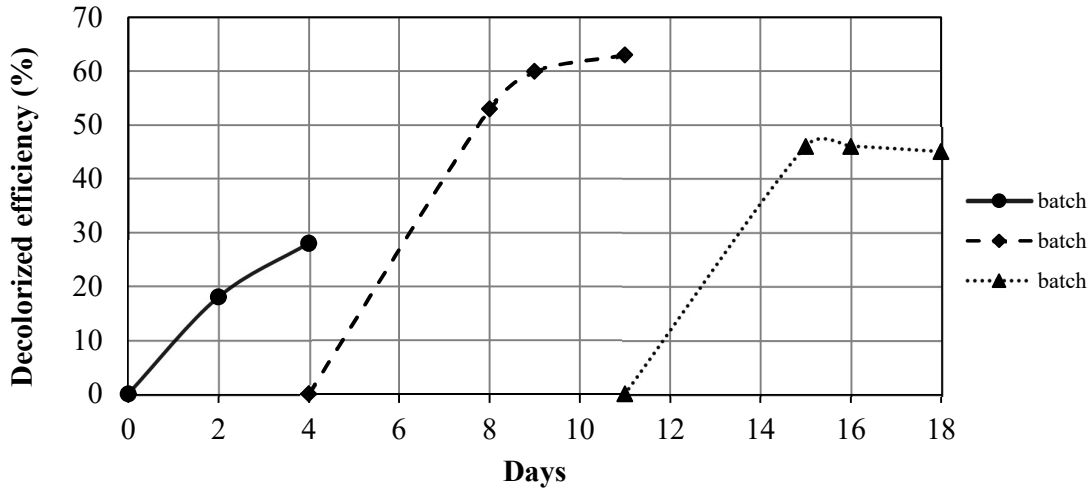

Fig. 4: Decolorized efficiency of immobilized *P. tephropora* in bioreactor.

However, the decolorized efficiency of *T. sanguinea* was decreased after 6 days because the color of textile wastewater was darkened. Téllez-Téllez et al. (2016) reported that *T. sanguinea* could synthesize a salmon red color pigment, cinnabarin, cinnabarinic acid, tramesanguin, and it led to the darker color, thus, the final experiment (decolorization of wastewater in bioreactor) was only tested by *P. tephropora*.

Decolorization of Wastewater in Bioreactor

The decolorized efficiency and repeated batch of textile wastewater treatment in the bioreactor were observed. The result showed that three repeated batches were carried out by the immobilized *P. tephropora*. In the first batch (initial color as 361 ADMI), the decolorized efficiency was 27.8% with a color of 260 ADMI (Fig. 4). In the second batch (initial color as 457 ADMI), the decolorized efficiency was 63.4% with a color of 167 ADMI. In the third batch, (initial color as 417 ADMI), the decolorized efficiency was 46.2% with a color of 224 ADMI. It was notable that the decolorized efficiency in the third batch slightly decreased, and it indicated that the decolorization of immobilized *P. tephropora* began inefficiently. For ligninolytic enzyme activities, laccase and MnP activities were increased but LiP activity was not detected (Table 3).

The relationship between the decolorized efficiency and ligninolytic activities was considered. It appeared that the decolorized efficiency did not correspond to the enzyme activity. The highest decolorized efficiency was received in the second batch and decreased in the third batch, the highest laccase and MnP activities happened in the third batch. The explanations of this inconsistency were (1) the production of enzyme isoforms with different affinity to dyes and substrates used in the reaction assays, (2) the restricted distribution of enzymes in the culture medium, and (3) other enzymes (such as cytochrome P450) occurred in the degradation process was not measured (Anastasi et al. 2010).

Table 3: Ligninolytic enzyme activity in bioreactor.

No. of batch	Ligninolytic enzyme activity (U/L)		
	Laccase	MnP	LiP
1st batch	11.5	25.6	0.0
2nd batch	15.5	85.9	0.0
3rd batch	113.0	445.1	0.0

CONCLUSIONS

A total of 11 isolates of white rot fungi were examined for the decolorized textile wastewater. Both *T. sanguinea* and *P. tephropora* were the fungal isolates with the highest potential for decolorization at 68.6% and 67.5%, respectively. Loofah was the best media for fungal immobilization and 120 C/N ratio was the optimal ratio for the decolorization. For the decolorized textile wastewater in a bioreactor, three repeated batches were carried out by the immobilized *P. tephropora*, and the highest decolorized efficiency was received in the second batch at 63.4%.

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