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Khor Choo Shan and Siti Roshayu Hassan

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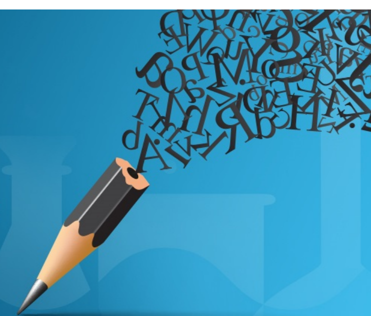

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Extraction of Crude Enzymes from Spent *P. ostreatus* Substrate and its Potential Use in Dye Removal

Khor Choo Shan [1, a)] and Siti Roshayu Hassan[1, b)]

[1]*Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan, Jeli, Malaysia*

[a)]cshann1230@gmail.com
[b)]Corresponding author: roshayu.h@umk.edu.my

Abstract. The application of enzyme in dye removal attracted considerable attention and shown its potential in recent years but the low stability and high production cost of enzyme are the key factors of the application. Tap water was used as solvent to extract the crude enzyme in this study to reduce the production cost. In order to optimise the yield of crude enzyme extract from spent *P. ostreatus* substrate, parameter such as suspended time duration of spent *P. ostreatus* substrate in tap water, agitation time and speed in incubator shaker were investigated. The effect of dye concentration, enzyme concentration, pH and temperature in Methylene blue and Rhodamine B removal using crude enzyme extracted were investigated. Bradford test was done to determine the presence of protein in the crude enzyme extracted and Fourier Transform Infrared Spectroscopy (FTIR) was used to determine the presence of functional group in crude enzyme extract. As a result, the optimum parameter to yield crude enzyme is 120 minutes of suspended time, 90 minutes of agitation time at 200 rpm agitation speed. Methylene blue shown the highest dye removal efficiency at 84.23% and Rhodamine B shown low dye removal efficiency <17%. Crude enzyme extracted from spent *P. ostreatus* substrate is efficient to remove Methylene blue but not efficient to remove Rhodamine B.

INTRODUCTION

In Malaysia, an average farm producing 100 ton of fresh mushrooms per annum generated approximately 438 ton of spent mushroom substrate. Each mushroom cultivation cycle lasts for 5 to 6 months and spent mushroom substrate will be disposed after this period. Approximately 5 kg of spent mushroom substrates are generated for each kg of mushrooms produced [1]. This huge amount of spent mushroom substrate is unsuitable for reuse in mushroom production. The current disposal strategy of spent mushroom substrate in Malaysia is by burning, spreading on land, burying, composting with animal manure, or landfilling [2]. In order to have a better solution on the management of spent mushroom substrate, there are a few applications already done by other scientists and researchers. According to Hanafi et al., [3], there are different application of spent mushroom substrate such as animal feedstock, fertilizer, energy generation and wastewater treatment.

Recently, low stability and high production cost are the key factors for the application of enzyme in dye removal. According to Munari et al., [4], lignin-degrading enzyme contained in white rot fungi which include *Pleurotus Ostreatus* have its ability to degrade a wide variety of dyes. Laccase are mostly reported that it is efficient in dye removal but laccase is low in production and this will cause the high production cost. Manganese-peroxidase (MnP) and lignin-peroxidase (LiP) have the same problem too. Besides that, xylanase have high yield of production from spent mushroom substrate but its efficiency in dye removal still need further investigation. In this study, crude enzyme will be extracted instead of a single enzyme since all the mentioned enzymes present in spent mushroom substrate have their potential uses in dye removal. The crude enzyme will undergo optimisation by altering few parameters to increase the yield of production and investigate the dye removal efficiencies of crude enzyme extracted.

Factors affecting the yield of enzyme extracted are the suspended time duration of spent *Pleurotus ostreatus* substrate in buffer, agitation time and speed of the sample which used to extract crude enzyme. Factors affecting the

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dye removal efficiencies included dye concentration, enzyme concentration, pH and temperature. Methylene blue and Rhodamine B are dyes selected to be investigate in this study.

MATERIALS AND METHODS

Extraction of Crude Enzyme

Approximately 10 g of collected spent mushroom substrate is suspend in 50 mL of tap water for 60, 90, 120 and 150 minutes and then agitated in incubator shaker for 60, 90, 120 and 150 minutes at 100, 150, 200 and 250 rpm at 25℃ respectively. Then, the sample will be filter through filter funnel and centrifuge at 10,000 × g at 4 °C for 20 minutes. The supernatant obtained was the crude enzyme extracted. Composition of crude enzyme extracted from every set of experiment were determined by Bradford Test.

Determination of Enzyme Concentration

Enzyme concentration was determined according to the Bradford protein determination method. Bovine Serum Albumin (BSA) was used as the standard protein to determine the protein concentration also known as enzyme concentration in this study. A standard BSA calibration curve was plotted by different BSA concentration from range 50 to 200 µg/mL with their respective absorbance value. 100 µL of crude enzyme extracted was added together with 1 mL of Bradford reagent into a cuvette and absorbance value was read at 595 nm using a spectrophotometer. Enzyme concentration can be calculated by using the linear equation obtained after the standard BSA calibration curve plotted.

Standard Calibration Curve Preparation

A standard calibration curve was prepared by reading absorbance value of different dye concentration range from 0.5 mg/L to 12.0 mg/L. From the calibration curve prepared, the final concentration of dye can be calculated after dye removal. Methylene Blue and Rhodamine B used in each experiment was diluted with distilled water from the prepared stock freshly according to the concentration required for each set of experiment.

Process Study

Volume of dye and enzyme used, concentration of dye and enzyme used, pH and temperature were investigated in this stage. These variables were investigated stage by stage to find the most efficient parameter in dye removal. For enzyme concentration, the crude enzyme extracted was considered as 100% concentration. The crude enzyme extracted was diluted with distilled water to get 20, 40, 60 and 80% concentration. pH value of the dye solution was altered by adding sodium hydroxide or hydrochloric acid until reach the required pH value. pH meter was used to read the pH value of dye solution. Hot plate was used to heat and monitor the temperature of dye solution at required temperature. All set of experiment was done triplicate to get a more accurate result. Duration of each set of dye removal experiment was 2 hours. Absorbance reading was record in every 30 minutes. Absorbance reading were measure using spectrophotometer at 663 nm for Methylene Blue and 554nm for Rhodamine B. The efficiency of crude enzyme extracted in dye removal determined by comparing the result using a control set of experiment which were dye solution without crude enzyme. The efficiency of dye removal was calculated using equation 1:

$$\text{Efficiency of dye removal (\%)} = \frac{Initial\ absorbance - Final\ Absorbance}{Initial\ Absorbance} \times 100\% \quad (1)$$

Fourier-Transform Infrared (FTIR) Spectroscopy

Fourier-Transform Infrared (FTIR) Spectroscopy are used to investigate the presence of possible functional groups in a molecule. A number of absorption peaks will display on screen when sample detected and functional groups of the sample can be identified based on the peaks shown. In this study, the crude enzyme extracted will analysis using FTIR to identify the functional groups contained hence the relationship of functional groups of the enzyme and the effect of dye removal can be investigated.

RESULTS AND DISCUSSION

Extraction of Crude Enzyme

There were 3 different parameters in the crude enzyme extraction process in order to optimize the yield of the crude enzyme produced. Parameters include suspended time (ST) of spent *Pleurotus Ostreatus* substrate in solvent, agitation time (AT) and agitation speed (AS) of sample in incubator shaker (Table 1). Every set of experiment was done in triplicate for accuracy. After all the three parameter were altered, the best parameter investigated are 120 minutes of suspended time, 90 minutes agitation time at 200 rpm agitation speed. This parameter yields concentration of crude enzyme about 198.72 µg/mL.

TABLE 1. Absorbance value and concentration of crude enzyme calculated when best parameter was investigated

	Parameter		Absorbance	Concentration of crude enzyme,
ST, minutes	**AS, rpm**	**AT, minutes**	**value**	**µg/mL**
120	100	90	0.539	147.61
120	150	90	0.708	194.56
120	200	90	0.723	198.72
120	250	90	0.643	176.50

Dye Removal

Three parameters were investigated in this stage included dye concentration, enzyme concentration and pH. The efficiency of dye removal was determined by altering these parameters, the dye removal test was investigated separately stage by stage. Manipulated and constant variables and ratio of enzyme and dye were all stated in each stage. Methylene blue and Rhodamine B were selected dye used to perform dye removal in this project. Methylene Blue and Rhodamine B are both basic dye. Methylene blue is a common dye used by researcher to conduct dye removal so it was chosen to investigate the dye removal efficiencies of crude enzyme extracted in this study. Rhodamine B was selected to compare the dye removal efficiencies to Methylene Blue. After comparing the efficiency of dye removal of Methylene Blue and Rhodamine B, Methylene Blue show higher efficiency and thus only Methylene Blue was proceeded to fourth parameter which is temperature to further optimize the efficiency of dye removal. All test was done in triplicate and the average reading were obtained.

Effect of Dye Concentration

Effect of dye concentration were performed by using different dye concentration in the range from 1, 2, 3, 4 to 5 mg/L for both dye (Table 2 and Table 3). 1 mL of 100% concentration crude enzyme were used and the volume of dye used for each concentration were 10 mL. The ratio of enzyme and dye were 1:10. For Methylene Blue, 1 mg/L dye concentration gave the highest dye removal efficiency with 58.91% compared to other dye concentration while for Rhodamine B, 4 mg/L dye concentration gave the highest dye removal efficiency with 11.27%.

TABLE 2. Efficiency of Methylene blue removal using different dye concentration

Dye concentration, mg/L	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	**Final**	**Initial**	**Final**	
1	0.180	0.133	0.606	0.249	58.91
2	0.306	0.263	1.561	1.235	20.88
3	0.471	0.356	2.812	1.940	31.01
4	0.630	0.434	4.017	2.531	36.99
5	0.713	0.524	4.647	3.214	30.84

Based on the result of Methylene Blue, the efficiency of dye removal increase when the lower dye concentration used. Amount of dye increase when dye concentration increase and cause the efficiency of dye removal decrease with the increase of dye concentration according to Wanyonyi, W. C et al., [5]. Other than that, lower dye concentration indicates higher dye removal efficient is possibly related to the maximum capacity of enzymes at a certain concentration for removing the dyes [6].

TABLE 3. Efficiency of Rhodamine B removal using different dye concentration

Dye concentration, mg/L	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	Final	Initial	Final	
1	0.207	0.197	0.775	0.714	7.87
2	0.400	0.385	1.955	1.864	4.65
3	0.587	0.550	3.099	2.873	7.29
4	0.772	0.694	4.231	3.754	11.27
5	0.913	0.825	5.093	4.555	10.56

Based on the result of Rhodamine B, 1 mg/L of Rhodamine B has lower efficiency than 4 and 5 mg/L of Rhodamine B. According to Tan, K. A et al., [7], the actual amount of dyes removed by enzyme were increased with increase of dye concentration. In this case, it can be assumed that the low efficiency of dye removal in lower dye concentration happened due to the less amount of dye contained in lower dye concentration. Thus, the higher dye concentration has higher dye removal efficiency due to the same theory.

Effect of Enzyme Concentration

Enzyme concentration investigated were 20, 40, 60, 80 and 100% and the volume used was remained in 1 mL and the ratio 1:10 of enzyme and dye were remained constant. Based on the result, 20% enzyme concentration shown the highest dye removal efficiency of Methylene blue at 49.47% and others enzyme concentration shown dye removal efficiency lower than 40% (Table 4). For Rhodamine B, result shown 100% enzyme concentration has the highest efficiency 16.16% and others enzyme concentration have a range of efficiency from 14.50% to 15.72% (Table 5). According to previous study done by Wehaidy, H. R et al., [8], the dye removal efficiency higher when lower concentration of crude enzyme was used. The lower enzyme concentration which present a 64% of dye removal efficiency was higher than 21% of dye removal efficiency when higher enzyme concentration used stated in the previous study. This is because of the crude enzyme may compete with other proteins contained in the crude culture filtrate and bring the crude enzyme and other impurities into a crowded environment. Thus, the dye removal process might delay due to the crowded environment and competition among crude enzyme and impurities. Based on the result, different enzyme concentration does not have big effect towards the efficiency of Rhodamine B removal.

Effect of pH

To further improve the efficiency of dye removal of crude enzyme, effect of pH was investigated. pH value altered to 4, 7 and 9 which represented acidic, neutral and basic condition to investigate the effect of pH toward the dye removal efficiency. Based on the result of Methylene Blue (Table 6), pH 4 shown the highest efficiency of dye removal compared to pH 7 and pH 9 with their efficiency 71.49%, 64,13% and 47.18% respectively. This is due to the different enzyme contained in the crude enzyme extracted which have different optimum pH value. Laccase have its optimum pH at 3-4 [9], Xylanase have its optimum pH at 6 [10], Lignin peroxidase have its optimum pH at 3 [11] and Manganese peroxidase have its optimum pH at 4-5 [12]. Since these lignocellulolytic enzyme all have their optimum pH below 7, so pH 4 which represent the acidic environment have the highest efficiency of dye removal. Although these enzymes active in acidic environment, enzymes still work but not that active in neutral and basic environment so the efficiency of dye removal were decrease as the pH value higher. For Rhodamine B (Table 7), it does not show higher dye removal efficiency compared to previous stage and even become lower efficiency when pH was altered in this stage. The highest efficiency of dye removal at previous stage was 16.16% but after altered the pH, it decreased to 15.34%, 8.35% and 4.36% respectively for pH 4, 7 and 9. In conclusion, crude enzyme extracted is not efficient in Rhodamine B removal.

TABLE 4. Efficiency of Methylene blue removal using different enzyme concentration

Enzyme concentration, %	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	Final	Initial	Final	
20	0.175	0.138	0.568	0.287	49.47
40	0.175	0.146	0.568	0.348	38.73
60	0.175	0.153	0.568	0.401	29.40
80	0.175	0.160	0.568	0.454	20.07
100	0.175	0.147	0.568	0.356	37.32

TABLE 5. Efficiency of Rhodamine B removal using different enzyme concentration

Enzyme concentration, %	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	Final	Initial	Final	
20	0.742	0.638	4.047	3.411	15.72
40	0.742	0.646	4.047	3.460	14.50
60	0.742	0.638	4.047	3.411	15.72
80	0.742	0.644	4.047	3.448	14.80
100	0.742	0.635	4.047	3.393	16.16

TABLE 6. Efficiency of Methylene blue removal using different pH

pH	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	Final	Initial	Final	
4	0.275	0.150	1.326	0.378	71.49
7	0.178	0.128	0.591	0.212	64.13
9	0.168	0.136	0.515	0.272	47.18

TABLE 7. Efficiency of Rhodamine B removal using different pH

pH	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	Final	Initial	Final	
4	0.772	0.666	4.231	3.582	15.34
7	0.787	0.728	4.322	3.961	8.35
9	0.793	0.762	4.359	4.169	4.36

Effect of Temperature

Due to the high potential of Methylene blue in dye removal which show high percentage in dye removal efficiency, temperature was altered to 40, 50 and 60°C to further investigate the efficiency of dye removal. The effect of temperature was performed using 1 mg/L dye concentration, 20% enzyme concentration and under pH 4 which represent acidic condition. The volume of enzyme and dye used were remained constant at the ratio 1:10. Based on Table 8, 84.23% of dye removal efficiency when temperature altered to 40°C. When temperature increased to 50 and 60°C, the dye removal efficiency decrease to 38.36% and 22.26% respectively. This is due to the optimum temperature of enzymes contained different. Optimum temperature for Laccase is 50°C [9], for Xylanse is 50°C [10], for Lignin peroxidase is 37°C [13] and for Manganase peroxidase is 25°C [12]. 40°C shown the highest efficiency in dye removal due to the temperature was not remained constant during experiment. The temperature was adjusted by using hot plate. During the experiment, the temperature has ±3°C difference since the temperature cannot be constantly fixed. Although laccase and xylanase both have optimum temperature at 50°C, but due to the error happened the enzyme may lose its activity when the temperature exceeds their optimum temperature. In conclusion, 1 mg/L dye

concentration with pH 4 in 40°C and 20% enzyme concentration gives the highest dye removal efficiency for Methylene Blue. The crude enzyme extracted is considered efficient in Methylene Blue removal.

TABLE 8. Efficiency of Methylene blue removal using different temperature

Temperature, °C	Absorbance Reading		Concentration (Calculation), mg/L		Efficiency, %
	Initial	Final	Initial	Final	
40	0.188	0.114	0.666	0.105	84.23
50	0.142	0.126	0.318	0.196	38.36
60	0.176	0.159	0.575	0.447	22.26

Functional groups of Crude Enzyme

The functional group of raw spent *Pleurotus Ostreatus* substrate, crude enzyme extracted from spent *Pleurotus Ostreatus* substrate, sample before dye removal and sample after dye removal were all analysed using Fourier-Transform Infrared Spectroscopy (FTIR). Based on Fig. 1, the spectrum of raw spent *Pleurotus Ostreatus* substrate (a) samples shown distinct peaks of N-H stretching at 3330.46 cm^{-1}, N-H bonding vibrations at 1632.62 cm^{-1}, C-H bending at 1373.81 cm^{-1}, C-N stretching at 1318.96 cm^{-1} and 1032.72 cm^{-1}. 3330.46 cm^{-1} is assigned to wide and medium N-H stretching which are possible to represent aliphatic primary amine compound in between 3400-3300 cm^{-1}. In the frequency range 1600-1300 cm^{-1}, the peaks, 1373.81 cm^{-1} mainly provide information on the C–H-bending vibrations which methyl group may present. For peak with 1318.96 cm^{-1} which located between 1342-1266 cm^{-1} and another peak at 1032.72 cm^{-1} located between 1250-1020 cm^{-1} were both corresponding to C-N stretching and possible to be an amine compound [14, 15, 16].

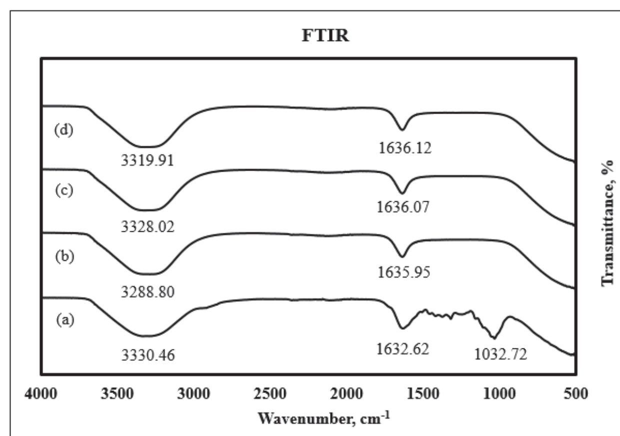

FIGURE 1. Fourier-Transform Infrared Spectroscopy (FTIR) result with wavenumber of main peak, (a) Raw Spent *Pleurotus Ostreatus* Substrate, (b) Crude Enyzme Extracted from Spent *Pleurotus Ostreatus* Substrate, (c) Before Dye Removal and (d) After Dye Removal

According to Wei, Z et al., [17], the band located around 3295 cm^{-1} represents N–H stretching vibrations that are mainly caused by proteins. Hydrogen bond present in a broad absorption band in the wavenumber between 3650 and 3250 cm^{-1}. The bands presence in the region confirms the presence of hydrate (H_2O), hydroxyl (-OH), ammonium, or amino group. It will follow by few spectra at frequencies of 1600–1300, 1200–1000 and 800–600 cm^{-1} if hydroxyl compound (-OH) present stated by Nandiyanto, A. B. D et al., [18].

Bands located between frequencies of 1700 to 1600 cm^{-1} were known as amide I. Amide I presence between the frequencies due to the C=O stretching vibration of the amide groups in weakly coupling with the in-plane N–H bending and C–N stretching and Amide I reflect the degree of hydrogen bonding. Hydrogen bonds in proteins are mainly located between peptide bonds. The higher the wavenumber, the weaker the H bonding which result a less-ordered protein structure. Hence, the amide I bands shifted to higher wavenumber will indicate the overall protein disordering. Other absorption bands between 1500 and 1000 cm^{-1} were known as "fingerprint" region [17].

In conclusion, the present of peak around 3295 cm^{-1} and between 1700 to 1600 cm^{-1} indicates the presence of protein in the sample. Based on the result obtained, all sample contained protein and protein was successfully extracted due to the present of related peak in crude enzyme extracted. The shifting of wavenumber among the samples in the same regions was mainly due to the bond length and force of the bond. Bond length decrease when the wavenumber of the peak shifted to higher wavenumber. The wavenumber of peak will shift to lower values if the bond length increase.

CONCLUSION

The crude enzyme extract from spent *Pleurotus ostreatus* substrate has successfully optimize by altering the suspended time of spent *Pleurotus ostreatus* substrate in solvent, agitation time and speed of sample in incubator shaker. The best parameter produced highest yield of crude enzyme extracted are 120 minutes suspended time, 90 minutes agitation time at 200 rpm.

Next, the ability of crude enzyme extracted in dye removal was proven when Methylene Blue was the selected dye. By altering dye concentration, enzyme concentration, pH value and temperature, the efficiency of Methylene Blue removal was improved to 84.23%. By comparing Methylene Blue and Rhodamine B, crude enzyme extracted shown its potential uses in Methylene Blue removal with 84.23% efficiency but it shown 15.34% efficiency only in Rhodamine B. In conclusion, the crude enzyme extracted from spent *Pleurotus ostreatus* substrate is not efficient in Rhodamine B removal but efficient in Methylene Blue removal.

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