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Potential Degradation and Kinetics of Melanoidin by Using Laccase from White Rot Fungus

Wittawat Toomsan[1,2,*], Pinthita Mungkarndee[1], Sophon Boonlue[3], Nguyen Thanh Giao[4], Sumana Siripattanakul-Ratpukdi[1]

[1] Department of Environmental Engineering, Faculty of Engineering, Khon Kaen University, Khon Kaen, Thailand

[2] Research Program on Development of Appropriate Technologies for Coloring Agent Removal from Textile Dyeing, Pulp & Paper, and Sugar Industries for Sustainable Management, Center of Excellence on Hazardous Substance Management (HSM), Chulalongkorn University, Bangkok, Thailand

[3] Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

[4] Department of Environmental Management, College of Environment and Natural Resources, Can Tho University, Can Tho, Vietnam

[*] Corresponding author: Email: ts.wittawat@kkumail.com

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Abstract

This study was attempted to use laccase extracted from white rot fungus to remove melanoidin in the ethanol production wastewater. The isolated fungus producing the highest laccase was identified as *Megaspororia* sp. The highest degradation efficiencies of the purified and crude laccases were 48.00% and 44.60%, respectively. Both degradation kinetics well fit Michaelis-Menten model. The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were 0.82% melanoidin and 0.0045% melanoidin h^{-1} for the degradation by the purified laccase and 0.71% melanoidin and 0.0037% melanoidin h^{-1} for the degradation by the crude laccase. Turnover number (K_{cat}) of purified and crude laccases were 0.00023 and 0.00019% melanoidin U^{-1} h^{-1}, respectively. Catalytic efficiency (K_{cat}/K_m) of purified and crude laccases were 0.00028 and 0.00027 U^{-1} h^{-1}, respectively. The affinity of the crude laccase was slightly higher because of its non-specificity. K_{cat} and K_{cat}/K_m of the purified laccase were higher than the crude laccase. Proposed potential degradation result showed that laccase could oxidize CH_3, carbonyl groups, haloalkanes (C–H), C–O and C–N bondings which probably caused decolorization of melanoidin in wastewater. Thus, the purified and crude laccases can be used to decolorize melanoidin-containing wastewater from ethanol industries. As the attempt to use purified laccase consumed times and costs especially in purification steps, the crude laccase can be used to degrade color of melanoidin in wastewater with only 3.4% lower than the purified laccase.

Keywords: Decolorization kinetics; Melanoidin; Laccase; Degradation; White rot fungi; *Megaspororia* sp.

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Introduction

Melanoidins are complex polymers that gave dark brown color in water and wastewater. They are occurred from non-enzymatic browning reaction between sugar and amino acid as known in Maillard reactions [1]. Melanoidins are widely distributed in several industries such as bakery, food, beer, coffee, sugarcane molasses and ethanol [1]. These industries produce a large volume of effluent and contain high strength wastewater in term of chemical oxygen demand and color which affected the environment. The conventional treatments, such as anaerobic and activated sludge treatments cannot reduce color in melanoidin-containing wastewater due to its complexity. The several studies reported various methods, such as coagulation-flocculation, ozonation and oxidation to reduce color from melanoidin [2]. However, these methods are too expensive to treat the wastewater [2].

Among the methods, biological treatment using white rot fungi was an effective technique and consumed lower cost for the wastewater treatment [3]. White rot fungi are eukaryotic microorganisms which grow on living and dead tree trunks by degrading cellulose and lignin with high efficiency. The use of white rot fungi was examined from previous study. For instance, white rot fungus *Pleurotus ostreatus* can remove sulfonated phenylazonaphthol dyes [4]. The fungi *Pleurotus ostreatus* and *Trametes versicolor* were used in a packed-bed reactor for decolorization of textile wastewaters [5]. The white-rot fungus *Bjerkandera adusta* was used to apply in bioreactor to degrade hexachlorocyclohexane [6] White rot fungi are applied in wastewater treatment system due to they can particularly produce ligninolytic enzyme, such as lignin peroxidase, manganese peroxidase and laccase which are highly effective to degrade lignin at β-O-4 and β-1 bonding [7–9]. Generally, the use of microorganism in wastewater treatment that still obtained more processes to remove microorganism from effluent. Within this context, enzyme utilization for wastewater treatment has been gaining the attention. Previously, several enzymes were studied and successfully applied to degrade the pollutant.

Among the enzymes, laccases are copper-containing phenol oxidase enzymes that are known as an oxidoreductase. Laccases appear to be involved in several processes of environmental treatment, such as detoxification of pollutants, pathogenicity and decolorization of wastewater [10]. Laccase presented its ability to degrade variety of compounds more than other peroxidases [11]. Therefore, melanoidins which are complex compounds contained several functional groups, can be degraded by laccase which has flexibility in degradation. Thus far, there was no publication focusing on the use of laccases to treat melanoidin-containing wastewater from ethanol production industries.

The objectives of this study were to extract and purify laccase from white rot fungus, to degrade melanoidin and examine its potential degradation and kinetics using purified and crude laccases. Previously, only few studies reported laccases from local white rot fungi to treat melanoidin-containing wastewater. This study is the first report of laccase from *Megasporoporia* sp. which was collected from mushroom in Kanchanaburi forest, Thailand in degrading melanoidin in wastewater and also its degradation kinetics. The melanoidin degradation and its kinetics from local white rot fungi in this study will be useful as a baseline information for academics and practitioners for designing and operation of wastewater treatment in the future.

Materials and methods

1) Isolation and screening of white rot fungi

Twenty-eight mushrooms that grew on living tree trunks, dead tree trunks and soil, were collected from evergreen forest, Kanchanaburi, Thailand (UTM Zone 48, N 14.841922, E 98.951560) in the rainy season. The fungal strains were isolated from fruiting bodies and

preserved on potato dextrose agar (PDA) at 4 °C for further experiments. The screening for potential laccase production fungal were performed by culturing in 1% of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) agar (plate assay). Positive fungi were cultured in sugarcane bagasse under solid state fermentation for 7 days at 30 °C. Then, the fungus which produced the highest laccase activity, was selected for further experiments.

2) Fungal identification

The fungus was also identified by analysing the internal transcribed spacer sequence of its nuclear ribosomal DNA (ITS rDNA) [12]. The ITS gene was amplified using a pair of primers; ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATCG-3'). Amplification was performed using the GENE Q Thermal Cycler model TC24H/(b) (Bioer technology Co., Ltd., Hangzhou, China) using the following cycling parameters; initial denaturation at 95 ºC for 5 min; followed by 35cycles of denaturation at 95 ºC for 1 min; annealing at 52 ºC for 1 min, and extension at 72 ºC for 1.5 min, and a final extension at 72 ºC for 10 min. Next, the PCR product was purified using a Qiagen PCR Purification Kit (Qiagen Co., Ltd., Tokyo, Japan) and then sequenced at First BASE Laboratories Sdn Bhd, Selangor, Malaysia. The DNA sequences were submitted to GenBank for homology analysis using the BLASTN program. Sequence alignment was performed using the CLUSTALX program [13]. The nucleotide sequence was deposited into the DNA Data Bank of Japan (DDBJ accession number LC269926).

3) Laccase extraction and purification

After fermentation, crude laccase was extracted by adding 50 mL of 0.1 M McIlvaine buffer (pH 8.0) [14] then shake at 150 rpm at 30 ºC for 60 min. The mixtures were centrifuged at 8,000 rpm for 15 min at 4 ºC. Purified laccase was carried out with a series of two steps which were precipitation by using 80% ammonium sulphate and gel filtration by using sephadex G-100 [15]. Crude and purified laccase were determined activity under laccase assay condition. Crude and purified laccase were preserved at –40 ºC for further experiments.

4) Laccase assay

Laccase activity was determined by measuring the ABTS degradation at 420 nm ($\varepsilon = 36000\ M^{-1}\ cm^{-1}$). The reaction mixture contained 100 µL 0.5 mM ABTS, 850 µL 0.1 M McIlvaine buffer (pH 4.0) and 50 µl culture supernatant. One unit (U) of enzyme activity was defined as the amount of enzyme oxidizing 1 µM of substrate per minute [16].

5) Preparation of melanoidin wastewater

The preparation of melanoidin wastewater was adapted from Georgiou et al. [1]. Melanoidin wastewater was prepared from wastewater taken from an ethanol industrial plant, Khok Sa-at Sub-district, Phu Khiao District, Chaiyaphum, Thailand. The raw vinasses wastewater contained color index of 12,500 ADMI and pH of 4.0. The vinasses wastewater was centrifuged at 8,000 rpm for 15 min and filtered using syringe filter (0.45 μm). The filtered solution was sterilized for 15 min at 121 ºC before using in the experiments.

6) Decolorization kinetic study of melanoidin by laccase

The decolorization kinetic study of melanoidin was carried out by varying the melanoidin concentrations. The total volume of solution was at 250 mL contained 1, 5, 10, 15 and 20% (v/v) of melanoidin, 20 units of the purified and crude laccases were used and adjusted with 0.1 M McIlvaine buffer (pH 4.0). The control sample was prepared by denaturing laccase under heating at 100 °C [17]. The degradation was carried out at 35 ºC in rotating mixer (150 rpm)

for 96 h. Each sample was collected with an interval of 12 h (0, 12, 24, 36, 48, 60, 72, 84 and 96 h). The decolorization of melanoidin was carried out by the UV–Vis Spectrophotometer at 475 nm and color index as ADMI (American Dye Manufacturers Institute) [1]. Each sample was calculated the decolorization efficiency (DE) as the following Eq. 1.

$$DE\ (\%) = \frac{D_i - D_e}{D_i} \times 100 \qquad \text{(Eq. 1)}$$

Where D_i = initial absorbance/ADMI and D_e = final absorbance/ADMI

The samples were preserved at –40 ºC for further experiment. Means of decolorization of five melanoidind concentrations were obtained from triplicated experimental samples. The experimental data as a scatter plot along with estimation were plotted. The initial decolorization rates of each melanoidin concentration under same period were plotted and then the K_m and V_{max} values were calculated by using Lineweaver -Burk plot which is linear plot between 1/[V] and 1/[S].

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

Where V = initial decolorization rate, S = substrate concentration, Slope = K_m/V_{max} and Y intercept = $1/V_{max}$

7) Potential degradation of melanoidin by purified laccase

The potential degradations of melanoidin were studied by using Fourier-transform infrared spectroscopy (FT-IR) couple with universal attenuated total reflectance sensor (UATR) (Spectrum Two™, PerkinElmer, USA). FT-IR was used to observe the shifting of functional groups between control and treated samples after 96 h of treatment. In this study, only purified laccase was chosen to examine potential degradation of melanoidin. Each sample was denatured enzyme by heating at 100 °C for 1 min to stop the reaction and filtrated by 0.2 μm syringe. The control and treated samples were dried at room temperature before measurement. The changing of intensity peak was observed and compared between control and treated samples.

8) Statistical analysis

All experimental data presented are average values ± standard deviation calculated using Excel 2010. To compare decolorization efficiencies between purified and crude laccases, multiple comparison tests were performed with t-test (significance levels = 0.05). Differences of the decolorization efficiencies were calculated using a one-way ANOVA variance analysis ($p < 0.05$) followed by the Duncan's test for multiple comparisons using the Stata 14 (Stata press, USA).

Results and discussion

1) Selection and identification of white rot fungi

Twenty-eight fungal strains were isolated from fruiting bodies using PDA until pure culture was obtained. The fungal isolates were designated codes as KKU-LKNG-XX. After screening using ABTS plate, the result showed that three fungal strains presented dark-green colonies containing KKU-LKNG-04, KKU-LKNG-07 and KKU-LKNG-16 (Figure 1). Then, three fungal strains were cultured under solid state fermentation using sugarcane bagasse. The highest laccase activity was detected from a fungal isolate named KKU-LKNG-07. After that, this fungus was identified as *Megasporoporia* sp. with maximum sequence similarities of 100% by using the analysis of ITS rRNA gene. Figure 2 shows morphology of *Megasporoporia* sp.

Figure 1 Screening of laccase production by ABTS plate assay.

Figure 2 Morphology of selected mushroom, identified as *Megasporoporia* sp.

2) Decolorization of melanoidin by purified and crude laccases

Melanoidin are cause of dark-brown color in vinasse wastewater. The degradation of melanoidin can be decolorized for vinasse wastewater [3]. In this study, decolorization efficiency and kinetics were focused since color is an important parameter for industrial wastewater quality standard. The decolorization of melanoidin by purified and crude laccases from *Megasporoporia* sp. is shown in Figure 3. At low melanoidin concentrations, the decolorization of melanoidin-containing wastewater using both purified and crude laccases seems to be more effective. Purified and crude laccases can oxidize color of melanoidin of up to 48.00% and 44.60% at 5% concentration of melanoidin (v/v), respectively (Figure 3). In term of ADMI unit (for color measurement), purified and crude laccases can reduce color of melanoidin to 39.24% and 35.79% at 5% concentration of melanoidin (v/v), respectively (the highest efficiencies). Trends of color and melanoidin reduction were well correlated. Based on statistical analysis, purified laccase had significant higher ability to remove color than crude laccase in all experiments with different melanoidin concentrations (Figure 3). To compare decolorozation efficiencies, purified laccase well removed color from the experiments with 1, 5, and 10% melanoidin (insignificant difference) while the color removal efficiencies from experiments 15 and 20% melanoidin were lower (Figure 3). In term of the crude laccase, decolorization efficiencies of all experiments with different melanoidin concentrations were statistically different. This could state that the melanoidin concentration plays an important role on the colored wastewater treatment. The previous study, Georgiou et al. reported that commercial laccase from *T. versicolor* was used to decolorize melanoidin-containing wastewater. The highest decolorization reached maximum efficiency of 47% at 10% concentration of melanoidin by free laccase [1]. This indicated that the laccases from *Megasporoporia* sp. in this study showed comparable decolorization efficiency to the commercial laccase and potential for industrial practice. Therefore, this white rot fungus can be researched further for commercial purpose.

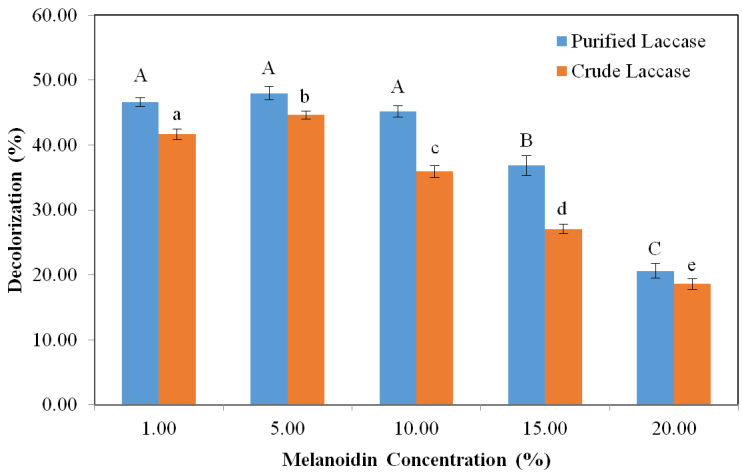

Figure 3 Decolorization of melanoidin by purified and crude laccases from *Megasporoporia* sp. during 4 days (Capital and lowercase letter represented the statistical analysis for the experiments with purified laccase, and crude laccase, respectively).

3) Kinetic study of melanoidin decolorization by laccases

The initial decolorized rates at different melanoidin concentrations were studied to find its kinetic values. For the kinetics applied in this study, a previous study reported kinetics of the enzymatic decolorization of textile dyes by laccase from *Cerrena unicolor* which presented K_m, V_{max} and K_{cat} using linear regression and HaneseWoolf plots [18]. Additional, Forootanfar et al. reported studies on the laccase-mediated decolorization, kinetic, and microtoxicity of some synthetic azo dyes which also demonstrated K_m and V_{max} using Lineweaver-Burk plot. Based on the previous works, the Michaelise-Menten equation is applied [19].

Table 1 shows kinetics of purified and crude laccases from *Megasporoporia* sp. in melanoidin degradation. The kinetic study showed that K_m and V_{max} values by the purified laccase were 0.82% melanoidin and 0.0045% melanoidin h^{-1}, respectively (Figure 4a). The kinetic study of melanoidin degradation by the crude laccase had K_m and V_{max} at 0.71% melanoidin and 0.0037% melanoidin h^{-1} (Figure 4b). Figure 5 demonstrates the comparison of the degradation kinetics of melanoidin-containing wastewater between purified and crude laccases. The crude laccase showed higher affinity and lower maximum rate of reaction than purified laccase. Vmax of purified laccase was higher than that of crude laccase due to its specificity. In term of K_{cat} and K_{cat}/K_m values, the K_{cat} values of purified and crude laccases were 0.00023 and 0.00019 % melanoidin U^{-1} h^{-1}. K_{cat} of purified laccase showed higher value than that of the crude laccase. This indicated that the purified laccase had higher turnover number than crude laccase. To compare the efficiencies of the different enzymes, the ratio K_{cat}/K_m was calculated to present an indication of each enzyme [20]. The K_{cat}/K_m values of purified and crude laccases were 0.00028 and 0.00027 U^{-1} h^{-1}. The catalytic efficiency of both laccases showed neary values.

Based on the kinetic parameters, the purification led to better melanoidin decolorization. However, the results from purified and crude laccases in this studies were not clearly different. This finding is agreeable to a previous work [17]. The phenol removal by crude Horseradish peroxidase was similar to purified Horseradish peroxidase. This is because presence of the significant quantity of proteinaceous matter in crude enzyme can be protected from inactivation [21]. Therefore, this crude laccase can be utilized instead of purified laccase.

Table 1 Kinetic parameters of purified and crude laccases from *Megasporoporia* sp. in degrading melanoidin

Enzyme	K_m	V_{max}	K_{cat}	K_{cat}/K_m
	% melanoidin	% melanoidin h^{-1}	% melanoidin U^{-1} h^{-1}	U^{-1} h^{-1}
Purified lacca se	0.82	$4.5x10^{-3}$	$0.23x10^{-3}$	$0.28x10^{-3}$
Crude laccase	0.71	$3.7x10^{-3}$	$0.19x10^{-3}$	$0.27x10^{-3}$

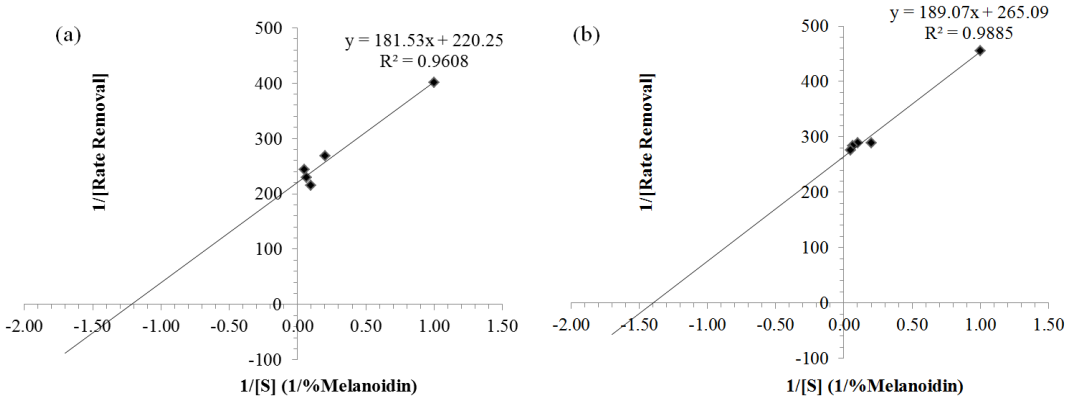

Figure 4 Lineweaver–Burk plot of decolorization kinetics using (a) purified laccase and (b) crude laccase.

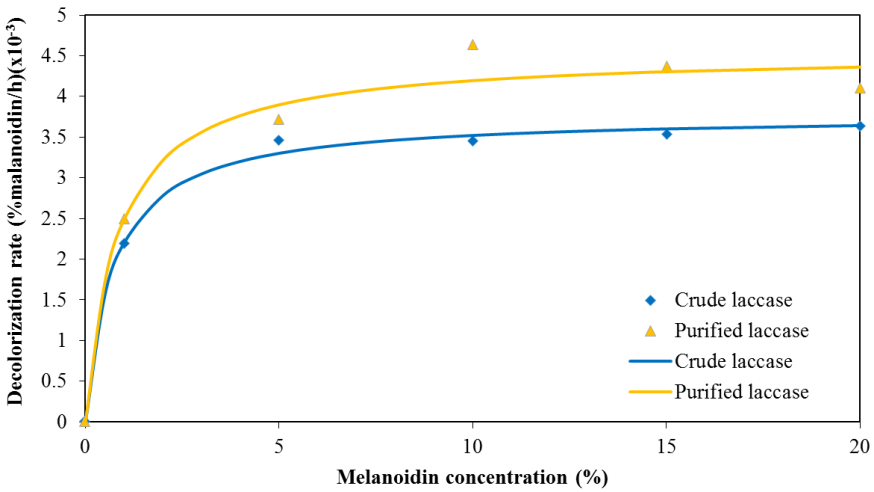

Figure 5 Decolorization kinetics of melanoidin wastewater by purified and crude laccases from *Megasporoporia* sp.

4) Potential degradation of melanoidin by purified laccase

FT-IR analysis was used to determine the depolymerization of melanoidin by the purified laccase. Figure 6 showed the changing of the functional groups between control and treated samples. The control sample was intense absorption between 3100 and 3600 cm^{-1}. The result showed the peak of O–H and N–H that were COOH and amides, respectively. The small peak was detected at 2985 and 2939 cm^{-1} indicating –CH and $–CH_3$ that implied to hydrocarbon chain. The intense peaks of 1711 and 1588 cm^{-1} correlated with carbonyl groups and amide (C=O) in primary amide functions due to the representation of C–O stretching of COO^- ketonic C–O and aromatic C–C conjugated with COO^-. The functional aliphatic C–H was observed at 1404 cm^{-1} and also haloalkanes (C–H) was detected at 1315 cm^{-1}. The C–O and aliphatic amine (C–N) bondings in a range of 1000 to 1100 cm^{-1} were found in controlled sample. The absorption peaks between

600 and 1000 cm^{-1} of control sample were similar to treated sample. As a result, the treated sample by laccase can be observed the reduction of C–H at 2958 cm^{-1} and carbonyl groups (C=O) at 1711 cm^{-1}. The haloalkanes (C–H) was degraded by observing at the wavelength of 1315 cm^{-1}. The C–O and aliphatic amine (C–N) bonding in a range of 1050 and 1100 cm^{-1} was obviously decomposed by purified laccase (Figure 6). This study supported to Kumar & Chandra (2018) [22], who studied the degradation of mela-noidin by using bacterial laccase, which showed the reduction of C–H, carbonyl groups and C–O bonding in a range of 1050 and 1100 cm^{-1}. The melanoidin degradation fate by fungal and bacterial laccases seem to be similar. However, the fungal laccase yield was commonly higher than the bacterial laccase. Only few studies reported that the useful of bacterial laccases were better than fungal laccases [23]. Therefore, the fungal laccase sounds potential for commercial purpose. In this study, the decomposed functional groups of melanoidin by fungal laccase may cause melanoidin decolorization in wastewater. The proposed mechanisms of functional groups degraded by laccase shown in Figure 7. This is supported by the reduction in peak showing in the spectra (Figure 6).

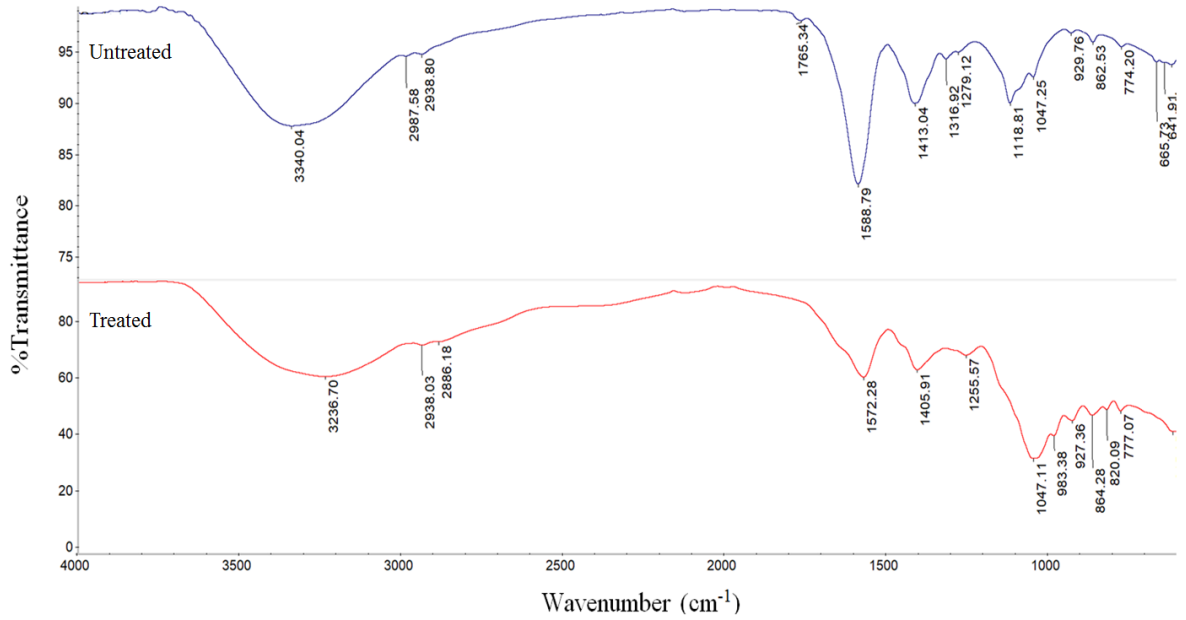

Figure 6 FT-IR spectra of melanoidin degradation by purified laccase from *Megasporoporia* sp.

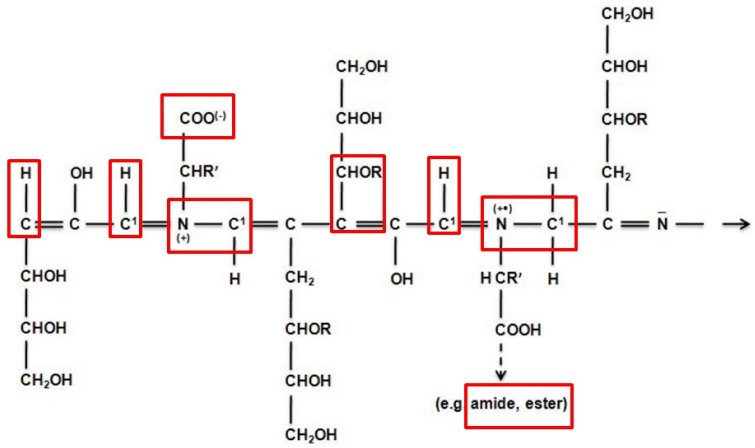

Figure 7 Proposed potential degradation of melanoidin polymer functional groups by laccase from *Megasporoporia* sp.

Conclusion

The purified and crude laccases extracted from *Megasporoporia* sp. which collected in Thailand can be reduced color of melanoidin. The purified laccase had higher decolorized efficiency than the crude laccase with 3.4% difference. In term of kinetic study, the purified laccase had K_m, V_{max}, K_{cat} and K_{cat}/K_m of 0.82% melanoidin, 0.0045% melanoidin h^{-1}, 0.00023 melanoidin U^{-1} h^{-1} and 0.00028 U^{-1} h^{-1}, respectively. The crude laccase had K_m, V_{max}, K_{cat} and K_{cat}/K_m of 0.71% melanoidin, 0.0037% melanoidin h^{-1}, 0.00019 melanoidin U^{-1} h^{-1} and 0.00027 U^{-1} h^{-1}, respectively. Laccase can oxidize CH_3, carbonyl groups and haloalkanes (C–H) resulting in decolorization of melanoidin.

Overall, laccase is promising for decolorization of melanoidin wastewater. The kinetic study showed that the purified laccase slightly better than crude laccase for degrading melanoidin wastewater. In practice, both crude and purified laccases could be applied. In the future, the operational and environmental parameters influencing decolorization of melanoidin wastewater, such as hydraulic retention time, enzyme dose, pH, co-contaminants should be investigated before the application.

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