

Lignin Peroxidase from the White-rot Fungus *Lentinus squarrosulus* MPN12 and its Application in the Biodegradation of Synthetic Dyes and Lignin

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Lignin peroxidase (LiP), which has been studied extensively in white-rot basidiomycetes and their potential to degrade dyes from textile wastewater, plays a role in the biodegradation of lignin from pulp and paper industry wastewater, as well as agricultural waste. Lignin peroxidase (LsLiP) was successfully purified from the newly isolated *Lentinus squarrosulus* MPN12 with a 47.1-fold purification and a 15.7% yield. After 48 h-incubation, LsLiP was able to decolorize all tested dyes up to 92% for Acid Blue 62 (NY3), followed by Porocion Brilliant blue HGR (PB, 73.5%), Acid Blue 281 (NY5, 70.5%), Acid Blue 113 (IN13, 61%), Acid red 266 (NY7, 56%), and 34.5% for Acid red 299 (NY1), compared to the negative control with the heat-denatured enzyme. The biodegradation potential of LsLiP was further suggested by the change of lignin structure based on Fourier transform infrared (FTIR) analyses. Lignin structure was noticeably changed before and after LsLiP treatment, especially in the fingerprint regions (1600 to 1000 cm⁻¹) and 2930 cm⁻¹ corresponding to the stretching vibrations of various groups in lignin. Thus, LsLiP has potential application in both enzyme-based decolorization of synthetic dyes and lignin biodegradation.

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INTRODUCTION

Lignin is a natural macromolecule having a cross-linked polyphenol structure (Garguak and Lebo 2000). The complex polymer structure contains phenolic hydroxyl groups, methoxy groups, and some terminal aldehyde groups in the side chains. The aromatic rings are abundantly present in lignin in the form of the basic phenylpropane units, which are syringyl, guaiacyl, and *p*-hydroxyphenyl (Zhang and Naebe 2021). They are responsible for maintaining the rigidity of the plant's cell wall. Thus, lignin is known to provide protection to plants against microbial and enzymatic attacks. With a persistent structure and difficulty in biodegradation, the presence of lignin and its derivatives in industries increases the pollution load on the environment. Indeed, lignin is present in abundance in pulp and paper industry wastewater, and agricultural waste (wheat straw, rice straw, and bagasse), etc. (Gaur *et al.* 2018; Pandit *et al.* 2021). Therefore, degrading lignin to reduce its negative impact on the environment is an urgent problem.

Lignin peroxidase (LiP, EC 1.11.1.14) is a ligninolytic enzyme, including manganese peroxidase (MnP, EC 1.11.1.13), lignin peroxidase, versatile peroxidases (VeP, EC 1.11.1.16), and laccase (Lac, EC 1.10.3.2). They are enzymes of biotechnological interest due to their ability to oxidize high redox potential aromatic compounds, including the lignin polymer. Lignin peroxidase was first discovered in *Phanerochaete chrysosporium* by Tien and Kirk (1983). It catalyzes the oxidative characteristics of a variety of non-phenolic and phenolic lignin aromatic complex compounds in the presence of H₂O₂ (Tien and Kirk 1984; Hamid and Rehman 2009), resulting in the cleavage of the C_α-C_β bond, aromatic ring opening, and phenolic oxidation (Christian *et al.* 2005).

Due to its high redox potential and enlarged substrate range, LiP is great for application in various industrial fields. For instance, LiP from the white-rot fungus *P. chrysosporium* plays a role in bio-pulping and bio-bleaching (Vandana *et al.* 2019). Several fungal species producing LiP enzymes, which are believed to play an important role in the biodegradation of lignin, are also known (Le Roes-Hill *et al.* 2011; Janusz *et al.* 2017; Vandana *et al.* 2019).

Lignin peroxidase is also known to be an essential enzyme in wastewater treatment. In principle, it could replace the use of chemical compounds for degrading paper waste; the reduced 5-fold concentration of lignin was observed after adding LiP enzymes in wastewater as compared with untreated wastewater, which is necessary due to the presence of lignin and its derivatives in pulp effluent increasing the pollution in the environment (Gaur *et al.* 2018). Furthermore, LiP is also known to play a role in the decolorization of dyes in textile industrial wastewater, which was also demonstrated by Parshetti *et al.* (2012) and Moubasher *et al.* (2017). It is predicted that the demand for LiP will increase in the future.

Currently, for biological treatments, various fungi show characteristics in the delignification of the lignocellulosic biomass and show potential for dye-decolorization due to their ligninolytic enzymes (Chia 2008; Yanto *et al.* 2019). Numerous ligninolytic enzymes have been isolated from fungal sources including white-rot fungi, such as *Phanerochaete chrysosporium* (Vandana *et al.* 2019), *Phanerochaete sordida* (Hirai *et al.* 2005), *Trametes versicolor* (Jönsson *et al.* 1994), brown-rot fungi such as *Coniophora puteana* (Irbe *et al.* 2014), and *Laetiporus sulphureus* (Mtui and Masalu 2008). However, among various fungi, the use of white-rot fungi *Lentinus squarrosulus* has been limited to application in the decolorization of dyes, and its role in the degradation of lignin has not been explored thoroughly.

Recently, Ravichandran *et al.* (2019) reported a versatile peroxidase from *L. squarrosulus* and its application in the biodegradation of lignocellulosic materials. However, the secretion of LiP by this white-rot fungus has not been comprehensively elucidated yet. Additionally, the application of this enzyme has not yet been developed properly due to the limited availability; the development of cost-effective production processes as well as purification is still difficult. Hence, in the present study, to gain insight into the redox potentials of LiP from the fungal system, the authors describe the production, purification, and characterization of a LiP from the newly isolated white-rot fungus *Lentinus squarrosulus* MPN12 (*LsLiP*) and its use for the bioconversion of lignin and different dyes.

EXPERIMENTAL

Materials and Methods

Fungal isolation and cultural conditions

The fungus (initially designated as MPN12) was collected from deciduous deadwood in the Muong Phang primeval forest (21°27'N, 103°09'E) in Dien Bien Province, Northwest Vietnam. The fungus was grown on a 2% malt-agar plate with antibiotic additions (0.005% streptomycin, penicillin). The seed culture was prepared by repeated sub-culturing on potato dextrose agar (PDA; Merck, Darmstadt, Germany) and stored at -80 °C for further experiments.

Molecular identification and phylogenetic analysis

For fungal identification, the ITS1–5.8S–ITS2 region was amplified from the genome of *L. squarrosulus* using a polymerase chain reaction (PCR) with a forward primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and a reverse primer ITS4: 5'-TCCTCCGCTTATTGATATTGC-3' (White *et al.* 1990). For the reaction, 12.5 µL of PCR Master mix kit (2X) (Thermo Fisher, Billerica, MA, USA), 3 µL DNA (deoxyribonucleic acid), 10 µM of each primer and nuclease-free water up to 25 µL were used. The thermal cycling conditions were 95 °C/3 min followed by 35 cycles of 94 °C/45 s, 55 °C/45 s, and 72 °C/45 s, and a final extension was performed at 72 °C/10 min. The amplification of the products was verified by electrophoresis using a 1.5% agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Next, amplicons were sequenced in an ABI Prism 3100 genetic analyzer (Applied Biosystems, Waltham, MA, USA). The sequences obtained were analyzed in the ChromasPro1.7.6 software (Technelysium, Brisbane, Australia) and compared with GenBank databases using the NCBI BLAST tool (Johnson *et al.* 2008).

Phylogenetic analysis

Phylogenetic tree analysis was performed utilizing the MEGA® v6.0 software (Pennsylvania State University, University Park, PA, USA) (Tamura *et al.* 2013). The alignment was carried out using Clustal W (European Molecular Biology Laboratory, Cambridge, CB10 1SD, United Kingdom) (Thompson *et al.* 1997), and the evolutionary history was inferred using the maximum likelihood method (ML) (Harris and Stöcker 1998) based on Kimura's two-parameter model (Kimura 1980) with 1000 bootstrap replicates. TreeView software (S&N Genealogy, Salisbury, United Kingdom) was used to edit evolutionary tree images (Page 1996).

Production and purification of lignin peroxidase

The production of the LsLiP of *L. squarrosulus* (strain MPN12) was performed in sterile plastic bags, each containing approximately 500 g of rice straw, and trace elements (KH₂PO₄ 0.5 g L⁻¹, MgSO₄ 0.25 g L⁻¹, (NH₄)₂SO₄ 1.5 g L⁻¹, CaCl₂ 0.05 g L⁻¹, and Na₂HPO₄·12H₂O 2.3 g L⁻¹). After two weeks of fungal growth, the proteins were extracted with distilled water. Then the mycelium and straw particles were removed by centrifugation (10,000 × g for 10 min) and filtration (filter GF6; Whatman PLC, Buckinghamshire, UK). The clear supernatant was harvested and concentrated by a tangential flow ultrafiltration system at 11 °C (10 kDa cut-off; Sartorius, Göttingen, Germany).

The enzyme purification was then performed by three steps of fast protein liquid chromatography (FPLC) using an ÄKTA Pure system (GE Healthcare, Danderyd, Sweden) with a detector operating at 280 nm. Firstly, the protein solution was applied to a HiTrap™ DEAE cellulose column equilibrated with 10 mM sodium acetate buffer (pH 4.5) and using a flow rate of 1 mL/min, eluted using a NaCl gradient of 0 to 1.5 M. LiP-active fractions were pooled and applied to Sephadex G-100 gel filtration column equilibrated with acetate buffer pH 4.5. The final purification step occurred on a HiTrap™ Q XL with 50 mM sodium acetate buffer (pH 5.5) and a NaCl gradient of 0.25 to 1.0 M at a flow rate of 0.5 mL/min. Pooled fractions with LiP activity were concentrated and desalted in the sodium acetate buffer (pH 5.5, 50 mM NaCl) using Amicon® Ultra centrifugal filters (10 kDa cut-off; Sartorius, Göttingen, Germany) and were stored at $-80\text{ }^{\circ}\text{C}$ for further use.

Enzyme activity assay

Peroxidase activity was assayed by using 2,4-dichlorophenol (2,4-DCP) (Sigma-Aldrich, Stockholm, Sweden) as the substrate. The reaction mixture (1 mL) contained 100 mM sodium acetate buffer (pH 5.5), 82 mM 4-aminoantipyrine (Sigma-Aldrich, Stockholm, Sweden), 20 mM 2,4-DCP, 40 mM hydrogen peroxide, and an appropriate amount of *LsLiP* (Yee and Wood 1996). The reaction was initiated by the addition of hydrogen peroxide, during the first 2 min of the reaction at $35\text{ }^{\circ}\text{C}$ at 510 nm. One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of substrate per min.

Enzyme characterization

The weight-averaged molecular weight (M_w) of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) (Laemmli 1970) at room temperature. The enzyme sample was incubated at $95\text{ }^{\circ}\text{C}$ for 10 min and centrifuged at 2000 rpm for 10 min. Analytical isoelectric focusing (IEF) was performed with the same electrophoresis system but using IEF precast gels (Novex IEF; Invitrogen, Massachusetts, USA). Staining of the protein bands was done using Coomassie Brilliant Blue R-250, which was followed by fixing and destaining.

The effect of pH on *LsLiP* activity was determined by conducting an enzyme assay within a pH range from 4.0 to 8.0 in 100 mM sodium acetate buffer (pH 4.0 to 5.5) and 100 mM sodium phosphate (pH 6.0 to 8.0). The optimal temperature was determined by measuring the enzyme activity at 20 to $55\text{ }^{\circ}\text{C}$. The temperature optimum was determined by following the oxidation of 2,4-DCP at different temperatures (20 to $55\text{ }^{\circ}\text{C}$) in sodium acetate buffer (100 mM) at pH 5.5. The pH stability of *LsLiP* was tested by incubating the enzyme activity at $35\text{ }^{\circ}\text{C}$ for different time periods in 100 mM sodium acetate buffer, pH 4.0 as well as in sodium phosphate buffer, pH 6.0 and pH 8.0. After 1 h-intervals of incubation, aliquots of the sample were taken, and the remaining LiP activity was measured as described above. The thermal stability of *LsLiP* was determined by measuring the residual activity after enzyme incubation at 4, 25, 40, and $60\text{ }^{\circ}\text{C}$ at an interval time of 20 min for up to 2 h. Furthermore, various metal ions, *e.g.*, Na^+ , K^+ , Ca^{2+} , Cu^{2+} , Zn^{2+} , Mg^{2+} , Ni^{2+} , and Fe^{2+} at 1 mM and 5 mM were used to elucidate their effects on the activity of *LsLiP*. All experiments were performed in triplicate.

Substrate specificity and kinetic analysis

Several phenolic compounds were tested as potential LiP substrates, including DCP, veratryl alcohol, guaiacol, ferulic acid, vanillic acid, and dimethoxyphenol

(concentration 20 mM). The rate of substrate oxidation was measured by determining the increase in absorbance at their wavelengths.

The Michaelis-Menten constant (K_m) and catalytic rate constant (k_{cat}) were determined by measuring the activity of *LsLiP* at several fixed concentrations of DCP (0.3 to 1.5 mM) at 35 °C for 2 min. The V_{max} and K_m parameters were calculated by applying the Lineweaver-Burk plot. Data correspond to the value \pm standard deviation (SD) of three independent experiments.

Lignin biodegradation

Lignin was extracted from rice-straw biomass, which was first pretreated with ultrasound irradiation (Sonic system SOMERSET) provided with the ultimate power of 500 W under different times (0, 10, 20, 30, and 40 min) in 2 M NaOH aqueous solution (1 g rice straw: 20 ml NaOH 2 M) at 90 °C. Then, the mixture was continuously (150 rpm) stirred at 90 °C for 1.5 h. Next, the mixture was washed with 0.1 M NaOH to separate lignin on the cellulose surface. After the filtration on a nylon cloth, the residue rich in cellulose was removed. The mixture of hydrolysates was then isolated to hemicellulose by precipitation of the acidified hydrolysate (pH was adjusted to 5.5 with HCl solution) with three volumes of 95% ethanol for 6 h. The pellets rich in hemicellulose were filtered and removed. As the last step, after the evaporation of ethanol, the alkali-soluble lignin was obtained by precipitation at pH 1.5 adjusted by HCl. The solid rich in lignin were then washed with acidified solution pH 2.0 and freeze-dried (Dinh *et al.* 2017).

A total of 10 mL of reaction mixture containing 0.1 g of lignin, 3.3 mL of ethanol (96%), 40 mM H₂O₂, and 25 U of purified *LsLiP* in 100 mM sodium acetate at pH 5.0 was incubated at 37 °C for 72 h with shaking at 150 rpm. The changes in lignin biodegradation were identified using Fourier transform infrared spectroscopy (FTIR) analysis, which was determined using a Nicolet iN10 FTIR Microscope (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a DTGS detector.

Table 1. Characteristics of Studied Dyes

Dye	λ_{max} (nm)	Type of Dye
Acid blue 113 (IN13)	580	Azo
Acid red 266 (NY7)	499	Azo
Acid red 299 (NY1)	520	Azo
Acid blue 62 (NY3)	595	Anthraquinone
Acid blue 281 (NY5)	600	Anthraquinone
Porocion Brilliant blue HGR (PB)	604	Anthraquinone

Dye decolorization

The decolorizing ability of 6 different dyes belonging to the azo and anthraquinone group by purified *LsLiP* was determined in the 1 mL-mixtures containing each dye at the final concentration of 0.15 mg/mL, 40 mM H₂O₂, and 0.25 U of purified *LsLiP* in 100 mM sodium acetate buffer (pH 5.0). Negative control was measured under the same conditions with the heat-denatured enzyme. After incubation at 25 °C for 12 and 48 h, decolorization was assayed by measuring the absorbance at the respective wavelength maximum of each dye (Table 1). Each decolorization experiment was performed in triplicate, and the mean of the decolorization percentages was reported. The percentage decolorization was determined using Eq. 1,

$$\text{Decolorization (\%)} = \frac{A_i - A_t}{A_i} \times 100 \quad (1)$$

where A_i is the initial absorbances of the mixture and A_t is the final absorbance of the mixture (Vandana *et al.* 2019).

RESULTS AND DISCUSSION

Identification of Fungal Strains Isolated with High LiP Activity

Identification of the fungal isolates was conducted through partial gene sequencing. Total DNA was extracted and amplified by PCR with primers (ITS1/ITS4). A sequence of 427 nucleotides in length was obtained and deposited in the GenBank nucleotides database (<http://www.ncbi.nlm.nih.gov/genbank/>) with the accession number MW879106. The sequence obtained was then compared with those available in the GenBank database by applying the NCBI BLAST tool. The results showed 99.76% similarities with the sequences of the *Lentinus squarrosulus* GU001951 species. Based on this genetic identification, the strain MPN12 was authenticated as *Lentinus squarrosulus* (Polyporaceae, Basidiomycota), which is a new species for Vietnam. Phylogenetic trees of fungi (ITS region) are shown in Fig. 1; the bootstrap values demonstrate the quality of the analyses.

Purification of LiP from *L. squarrosulus*

For purification studies, a large amount of *LsLiP* was produced under conditions of solid-state fermentation with rice straw as a growth substrate to obtain a crude extract with a total *LsLiP* activity of 3600 U (measured with DCP, present H_2O_2). Subsequently, LiP from *L. squarrosulus* was successfully purified using three-step column chromatography procedures including DEAE, Sephadex G-100, and HiTrap™ Q XL columns. At the first step of purification, the specific activity of *LsLiP* was increased 6.7-fold from 0.6 to 4 U mg^{-1} (Table 2) after separation by the anion exchanger DEAE-cellulose. Pooled fractions of total activity of 1250 U were subsequently applied to size exclusion chromatography (GE Healthcare, Danderyd, Sweden) with the Sephadex™ G-100 column.

Table 2. Purification Summary of *LsLiP* from *L. squarrosulus*

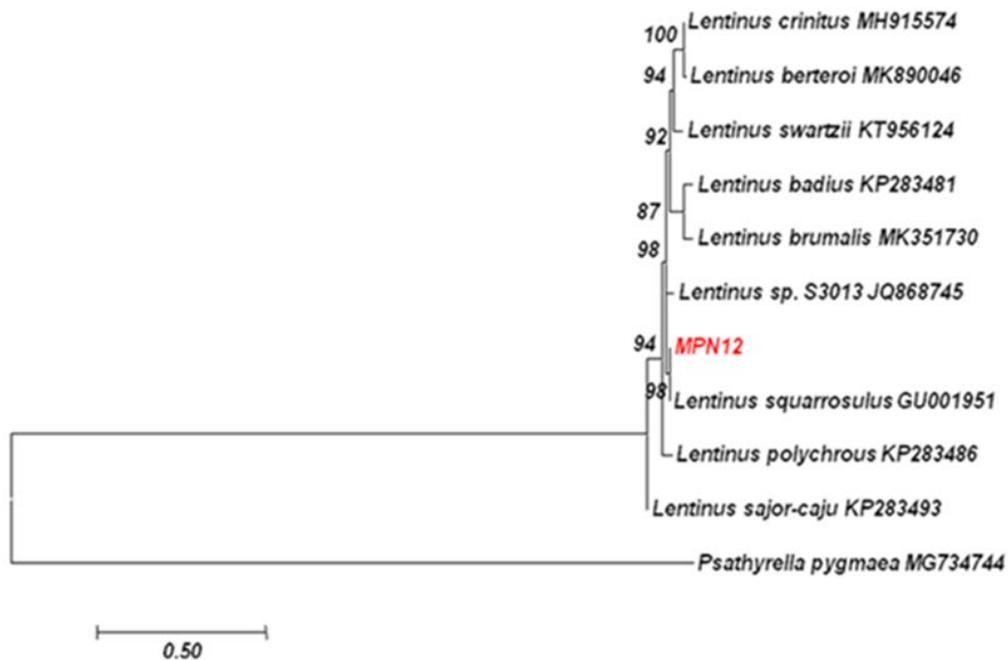
Purification Steps	Total Protein (mg)	Total Activity (U*)	Specific Activity, (U mg^{-1} *)	Yield (%)	Purification (Fold)
Crude Extract	5400	3600	0.6	100	1.0
DEAE Cellulose	350	1250	4.0	35	6.7
Sephadex G-100	80	831	10.4	23	17.3
HiTrap™ Q XL	20	566	28.3	15.7	47.1

Note: The enzyme activity was determined against 2,4-dichlorophenol (2,4-DCP) in 100-mM sodium acetate buffer (pH 5.5), at 35 °C

(a)

AAATTCTCAACCTAACGGGTTCTTAACGGGACTTGGTTACCTTGCCTCCTTGGTATT
 CCGAGGAGCATGCCTGTTTGAGTGTCATGTAAGTAATGTGAATTGCAGAATTCAGTG
 AATCATCGAATCTTTGAACGCGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
 AACGGATCTCTTGGTATCAGAATGTGTATTGCGATGTAACGCATCTATATACTT
 TCAGCTTCTTGCCTAGTTGTTACTGGGCCTACGTTTCACTACAAACACTTTTAAAGTG
 GGTTTCAGGAGCTTCGAAAGCGAGAAAGGGCCTTCACGGGCTTTCGAGGCATGTG
 CACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGCGGAAGGATCATTATCGA
 GTTTTGAAACGGGTTGTAGCTGGCCTTC

(b)



(c)



Fig. 1. Internal transcribed spacer (ITS) region was used to identify the fungal taxonomy (a), phylogeny tree was performed based on ML method using a partial rDNA-ITS sequence of the MPN12 fungal strain; *Psathyrella pygmaea* MG734744 was used as an outgroup taxon (b), and white-rot fungus *Lentinus squarrosulus* MPN12 (c).

These active fractions were further separated using a HiTrap™ Q XL column and a major peak of LiP activity was collected at a NaCl gradient of 0.5 mM (Fig. 2a). The LiP-active protein was purified with a total activity of 566 U with up to 47.1-fold purification and a 15.7% recovery (Table 2).

The purified *LsLiP* after the final step of HiTrap™ Q XL columns was assessed by 12% SDS-PAGE analysis, which appeared as a single band with a molecular mass of 55 kDa. Purified *LsLiP* revealed an acidic isoelectric pH value (pI) of 4.5 in the corresponding native IEF gel after staining with colloidal blue (Fig. 2b).

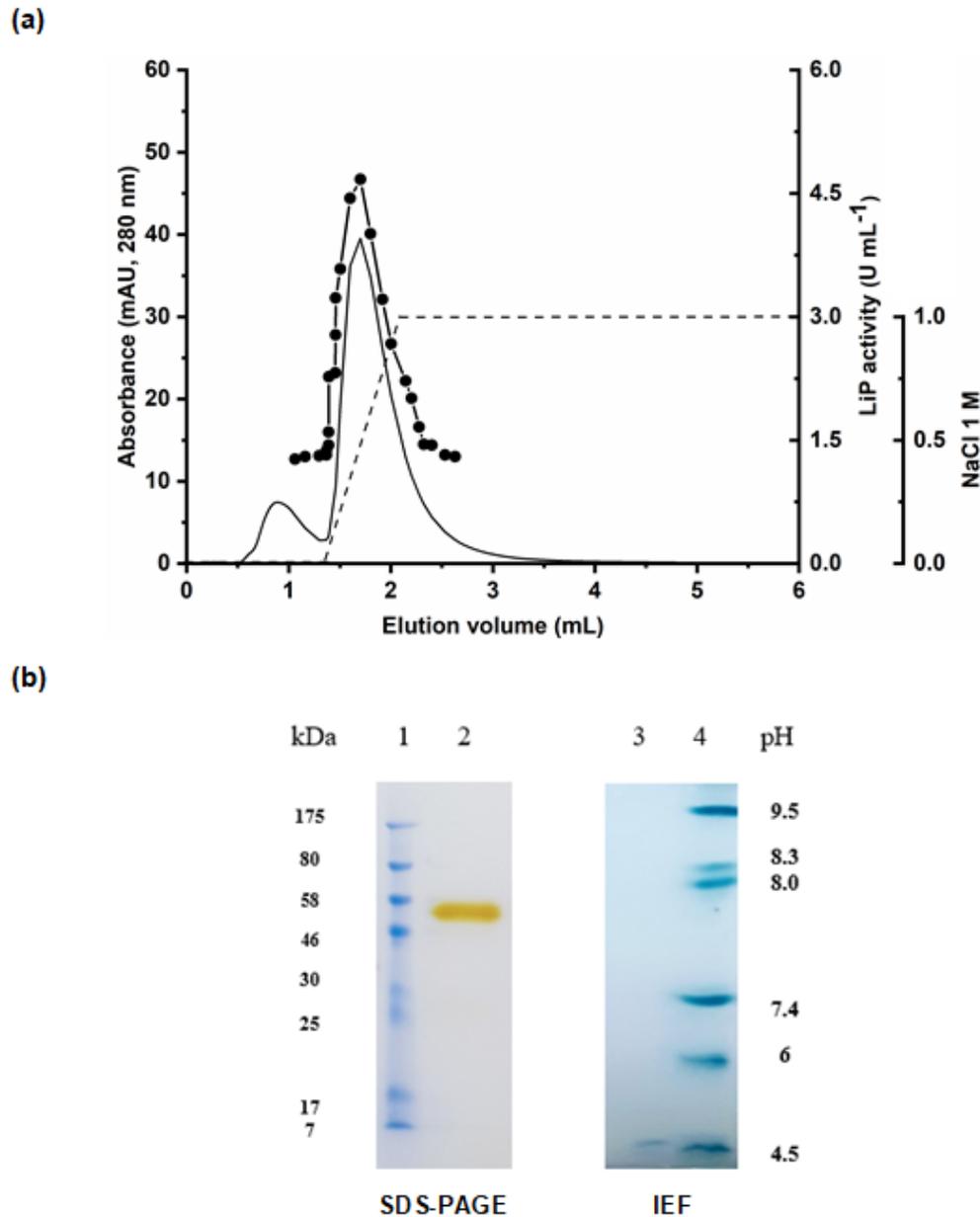


Fig. 2. (a) FPLC elution profile of the first and final purification steps of *LsLiP*: HiTrap™ Q XL column: absorbance at 280 nm (solid line), LiP activity (black circles), and NaCl gradient (dashed line) and (b) SDS-PAGE gel and IEF analysis of purified *LsLiP*: (lane 2,3), protein marker (lane 1,4)

Effect of pH and Temperature on the Activity of LsLiP

The optimal pH of *LsLiP* was determined to be between pH 4.0 and 8.0. The highest activity was observed at pH 5.0 in sodium acetate buffer, and the *LsLiP* lost its activity at pH 8.0 in sodium phosphate buffer (Fig. 3a). This optimal pH range of *LsLiP* is similar to those of other LiPs (Alam *et al.* 2009). The *LsLiP* exhibited optimal temperatures at 35 °C and approximately 10% of the maximum activity at 55 °C (Fig. 3b).

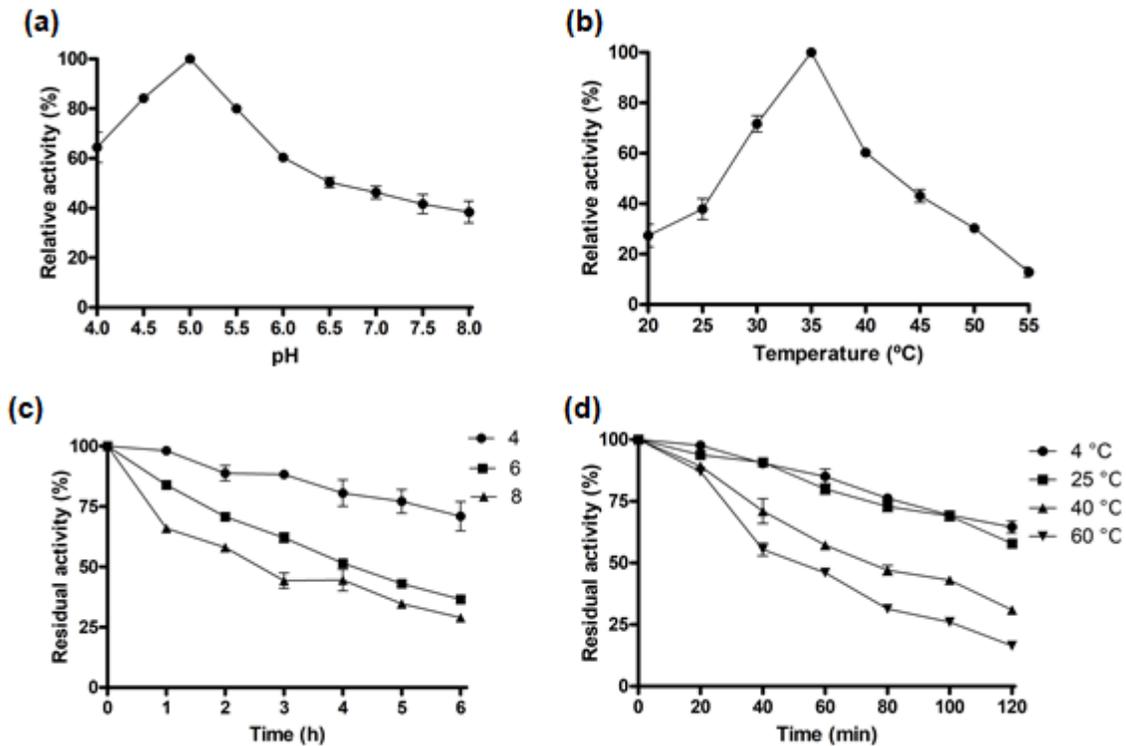


Fig. 3. Effects of pH and temperature on the activity of *LsLiP*: (a) Optimum pH: *LsLiP* was determined at various pH levels (pH 4.0 to 8.0) at 35 °C; (b) Optimum temperature: *LsLiP* activity was measured at pH 5.0 with different temperatures (20 to 55 °C); (c) pH stability: pH stability of *LsLiP* was determined by measuring the residual activity at 35 °C after incubation of the enzyme at pH 4, 6, and 8 for the indicated times; (d) Thermal stability: Thermal stability of *LsLiP* was determined by measuring the residual activity at pH 5.0 after incubation of the enzyme at 4 °C, 25 °C, 40 °C, and 60 °C for the indicated times. Each value represents the mean \pm SD of three measurements.

The stability of *LsLiP* at different pHs was determined by measuring residual activity at optimum conditions (pH 5.0 and 35 °C) after the incubation of the enzyme at various pH (4.0 to 8.0) and for the indicated times. The *LsLiP* maintained peroxidase activity at pH 4.0 range for 3.0 h and was gradually denatured at pH 6.0. At pH 8.0, *LsLiP* lost its activity rapidly (Fig. 3c).

The thermal stability of *LsLiP* was determined by measuring the enzymatic activity at the optimal operating conditions and incubation at various temperatures (4 to 60 °C) for the indicated times. The *LsLiP* maintained its activity at 4 to 25 °C for 1 h. However, at 40 °C, the activity decreased to 50% and 75% of the initial activity after 1 and 2 h, respectively. The *LsLiP* activity rapidly reduced at 60 °C (Fig. 3d).

Effect of Metal Ions on the LsLiP Activity

The investigation of the effect of different metal cations (1 and 5 mM) on peroxidase activity of purified LsLiP showed that its activity was slightly enhanced by adding divalent cations, *i.e.*, Cu²⁺, Fe²⁺, Ca²⁺, Zn²⁺, Mg²⁺, and Ni²⁺, at concentrations of both 1 and 5 mM. These results illustrated that divalent cations were required for the activity of LsLiP. This was consistent with previous studies. Louie and Meade (1999) showed that Fe²⁺ can coordinate with oxidative site residues, leading to the activation of enzymes, whereas Fodil *et al.* (2012) showed that Ca²⁺ and Cu²⁺ were required for the stabilization in the active site of peroxidases. However, the addition of monovalent cation did not affect LsLiP activity (Table 3).

Table 3. Effect of Metal Ions on LsLiP Activity

Metal Ion	Relative Activity (%)	
	1 mM	5 mM
None	100	100
Na ⁺	98.7	94
K ⁺	97.8	92
Cu ²⁺	169.3	210.8
Ni ²⁺	112.5	103.2
Ca ²⁺	108.3	108.5
Zn ²⁺	108.7	113.7
Mg ²⁺	105	107.3
Fe ²⁺	123.9	112.2

Note: Each value represents the mean of triplicate measurements and varies from the mean by no more than 3%

Substrate Specificity and Kinetic Analysis

Additionally, LsLiP showed a substrate preference for DCP among the phenolic compounds investigated (Fig. 4).

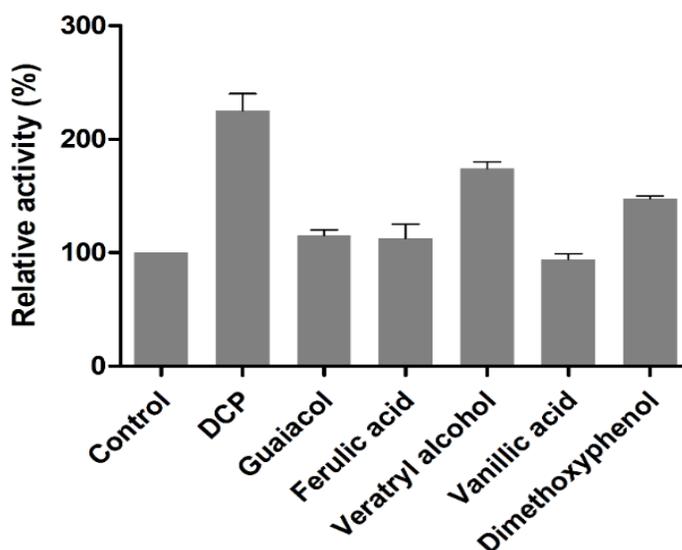


Fig. 4. Effect of the different substrates on the LsLiP activity

It showed up to 240% activity for DCP, which was followed by 180% activity for veratryl alcohol compared with that of the control. However, *LsLiP* showed much lower activity against other aromatic compounds (guaiacol, ferulic acid, and vanillic acid) (Fig. 4). Broad substrate, specifically, is commonly observed for most of the known LiPs, among which veratryl alcohol is the most frequently preferred (Vandana *et al.* 2019).

Kinetic constants of purified *LsLiP* were studied with DCP as substrates. Various concentrations of DCP ranging from 0.3 to 1.5 mM were plotted against the respective initial specific activities (V) of the purified *LsLiP*. A Lineweaver-Burk reciprocal plot was constructed, and the Michaelis-Menten kinetics yielded a hyperbolic curve (Fig. 5). The *LsLiP* showed a K_m value of 2.45 μM and a V_{max} value of 285 $\mu\text{mol}/\text{min}$. The catalytic rate (k_{cat}) value of the *LsLiP* peroxidase reaction was 12 s^{-1} , which led to a catalytic efficiency (k_{cat}/K_m) of 4.85 $\mu\text{M}^{-1} \text{s}^{-1}$.

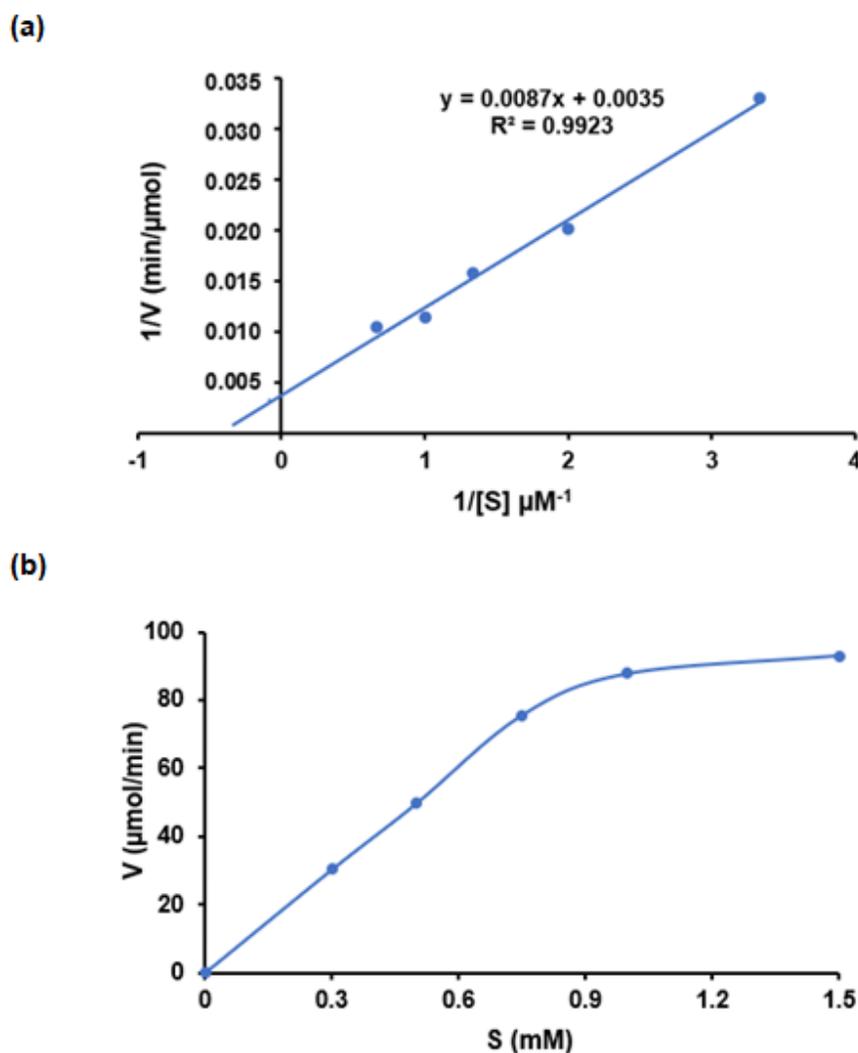


Fig. 5. (a) Lineweaver-Burk plot and (b) Michaelis-Menten plot for the purified *LsLiP*

Lignin Biodegradation by LsLiP

To determine the biodegradation of lignin by the *LsLiP* enzyme, the current authors used lignin derived from rice straw as a raw substrate (Dinh *et al.* 2017). The FTIR spectra of lignin before and after *LsLiP* treatment are shown in Fig. 6, and the major bands are assigned from a summary compiled from previous studies (Table 4) (Stark *et al.* 2016; Deng *et al.* 2019). The fingerprint region (1600 to 1000 cm^{-1}) in the spectra of lignin noticeably changed after using *LsLiP* treatment, corresponding to the stretching vibrations of various groups in lignin (Ying *et al.* 2018). This shows that the overall structure of lignin had been destroyed to a certain extent, after enzyme treatment. The stretching vibration of the C-O bonds in aliphatic alcohols and aliphatic ethers-derived aromatic ethers at 1115 cm^{-1} suggested that the band at 1115 cm^{-1} was caused by ether linkages adsorption. Next, the intensity of the peak at 1216 cm^{-1} changed noticeably, suggesting that the carbonyl in guaiacyl was interrupted during the treatment. The intensity of lignin at 1450 cm^{-1} decreased, indicating that the C-H of the aromatic ring in lignin could be deformed. This remarkable change at 1593 cm^{-1} was mainly attributed to the stretching of the aryl ring in lignin, and the peak intensity decreased after enzymatic treatment. The appearance of a peak of 1622 cm^{-1} was related to vibrations of aromatic rings present on lignin, which allowed for the prediction that the conjugated double bonds were generated.

Table 4. Assignments of FTIR Peaks in Lignin Samples

Wavenumber (cm^{-1})	Assignment
3200 to 3400	Stretching vibration of the hydroxyl group of hydrogen bond
2930	C-H stretching vibration of the methyl and methylene groups in lignin
1593, 1622	Ring stretching vibration peak of the benzene ring
1450	Deformation peak of C-H within vibration plane of aromatic ring skeleton
1216	The C-O stretching of G type lignin
1115	C-O stretching vibration in aliphatic alcohols and aliphatic ethers-derived aromatic ethers

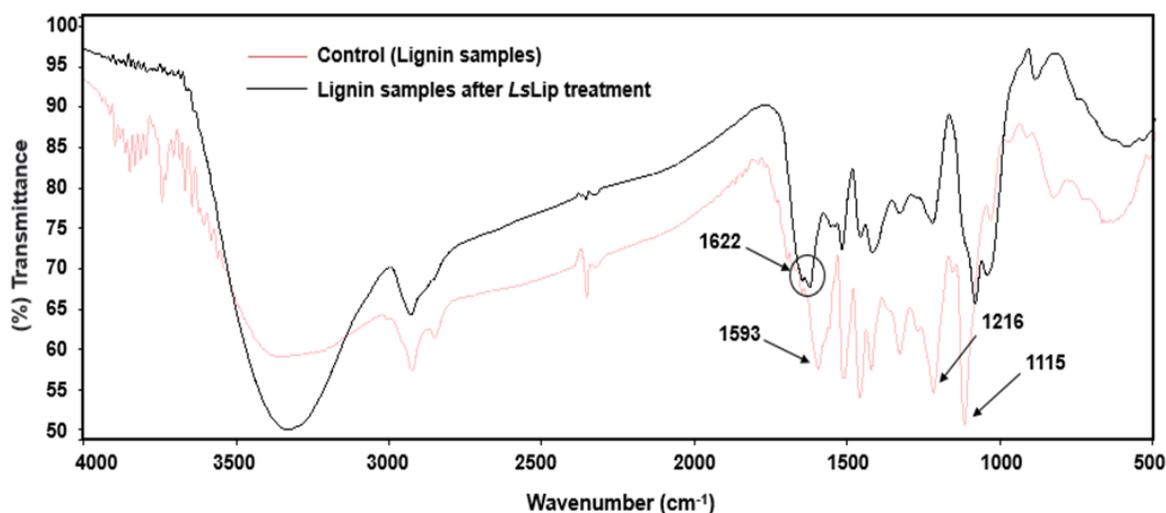


Fig. 6. FTIR spectra of lignin samples before and after *LsLiP* treatment

The intensity of the peak at 2930 cm^{-1} decreased noticeably after LiP treatment, which was mainly attributed to the C–H stretching vibration of the methyl and methylene groups in lignin. The band strikingly increased in the region 3200 to 3400 cm^{-1} , which was assigned to O–H stretching vibrations. This band was caused by the presence of alcoholic and phenol hydroxyl groups. Taken together, the FTIR analysis results of the present study indicated that the peak intensity of lignin structure properties remarkably changed after *LsLiP* treatment, elucidating the potential of *LsLiP* in the finely-tuned degradation of lignin polymers.

Dye Decolorization by *LsLiP*

The decolorization of six different dyes by using *LsLiP* was determined for 12 h and 48 h incubation, at pH 5.0. The results showed that the heat-denatured enzyme used as negative control clearly did not decolorize the tested dyes. Moreover, the decolorization increased with time incubation. In the decolorization of all the dyes at pH 5.0, NY3 was decolorized with higher efficiency than other dyes, while NY1 was difficult to decolorize. The decolorization of dyes was 63%, 50%, 54%, 20.5%, 42.5%, and 41% for NY3, NY5, PB, NY1, IN13, and NY7, respectively, at 12 h incubation. The decolorization level of all dyes remarkably increased to 92%, 70.5%, 73.5%, 34.5%, 61%, and 56% for NY3, NY5, PB, NY1, IN13, and NY7, respectively, at 48 h incubation (Fig. 7a). Moreover, Fig. 7b reveals that the color of the dye was removed after 48 h treatment in the order of NY3 > PB > NY5 > IN3 > NY7 > NY1. The decolorization level of azo dyes was lower than anthraquinone dyes. This could explain why even small structural differences in dyes can be expected to affect the decolorization level of dyes, which has also been investigated by Park *et al.* (2004).

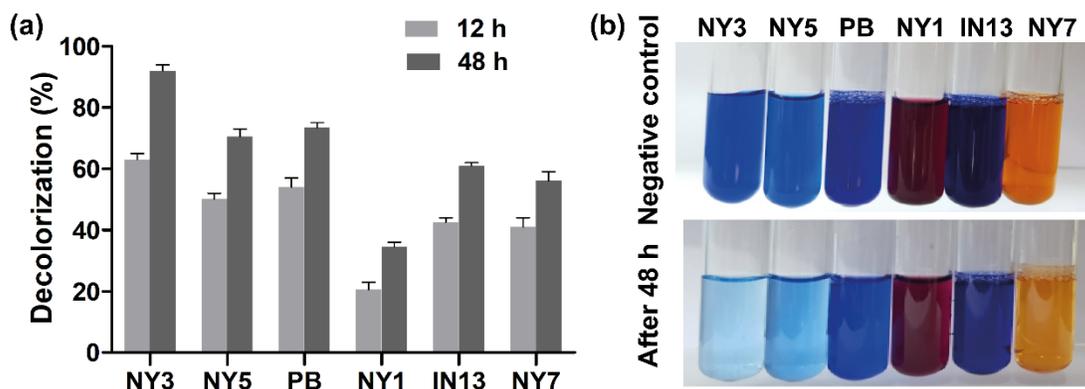


Fig. 7. Percentage decolorization of dyes by *LsLiP*

Discussion

The ligninolytic enzymes such as lignin peroxidase that are predominantly secreted by white-rot fungi are varied in the catalytic characteristics with the host species and their ecological recess. Accordingly, the present study reports the characteristics of a LiP purified from white-rot fungi *Lentinus squarrosulus*, which was isolated in the primeval forest, Vietnam. The enzyme was purified with 47.1-fold purification through anion and size exclusion chromatographic procedures. The purified protein showed a molecular weight of 55 kDa (Fig. 2b), which was slightly higher than LiPs from other fungi previously

reported (38 to 46 kDa) (Sahadevan *et al.* 2016; Chandra and Madakka 2019) but marginally lower than that from bacterial sources (66 kDa) (Ghodake *et al.* 2009; Parshetti *et al.* 2012; Patil 2014)

Lignin peroxidase was able to oxidize several phenolic compounds in a wide variety of applications. Among the aromatic compounds, DCP and veratryl alcohol as the main substrate of LiP have been studied the most. However, numerous previous studies have reported the kinetic characteristics of LiP using veratryl alcohol as the substrate (Ollikka *et al.* 1993; Koduri and Tien 1994; Lee *et al.* 2001; Casciello *et al.* 2017; Romero *et al.* 2019 Vandana *et al.* 2019), but only a few have reported the kinetics of DCP as the substrate (Yee and Wood 1997; Ryu *et al.* 2008). In this study, the authors showed a LiP from *L. squarrosulus* (*LsLiP*) that catalyzes the oxidation of DCP, veratryl alcohol, and other phenolic compounds. Of the different substrates tested, DCP was the substrate that was subjected to maximum oxidation by *LsLiP*. Accordingly, the kinetic parameters for the oxidation of DCP by *LsLiP* in the presence of H₂O₂ were investigated (Fig. 5). The kinetic data revealed a noticeable difference in the K_m value for DCP of *LsLiP* and other LiPs. Thus, the K_m value of *LsLiP* was 2.45 μM , which was lower than that of a bacterium *Streptomyces viridosporus* (372 μM) (Yee and Wood 1997), and white-rot fungus *P. chrysosporium* (359 μM) (Ryu *et al.* 2008). Moreover, the catalytic efficiency (k_{cat}/K_m) of *LsLiP* (4.85 $\mu\text{M}^{-1} \text{s}^{-1}$) was higher than that of LiP from white-rot fungus *P. chrysosporium* (6.34 $\mu\text{M}^{-1} \text{min}^{-1}$), indicating that *LsLiP* exhibits a strong affinity against this substrate. The pH profile revealed the optimum pH for DCP oxidation of *LsLiP* was in the acidic range as for other ligninolytic peroxidases (Couto *et al.* 2006; Fernández-Fueyo *et al.* 2014) and its enzymatic activity decreased rapidly beyond the optimal pH (Fig. 3a). The *LsLiP* maintained its activity at pH 4.0 and gradually lost its activity at pH 6.0 to 8.0 (Fig. 3c). Concerning thermal stability, a temperature higher than 40 °C was not beneficial for the enzyme. The *LsLiP* exhibited an apparent optimum activity at 35 °C, which was similar to the optimum physiological temperature. At 40 °C, the activity lost approximately 70% of its maximum after incubation for 2 h (Fig. 3d). This could be due to the structural Ca²⁺ ions of the enzyme that are lost at high temperatures (Ayuso-Fernández *et al.* 2018).

To ascertain the potential of purified *LsLiP*, the enzyme was employed to degrade the lignin and decolorize the tested dyes. Fourier-transform infrared spectroscopy analysis showed that lignin structure before and after *LsLiP* treatment was noticeably changed, especially in region 1600 to 1000 cm^{-1} . Moreover, the intensity of the peak at 2930 cm^{-1} changed slightly after *LsLiP* treatment, which was predicted to be the main cause of lignin cleavage. Therefore, *LsLiP* could degrade lignin in rice straw. This enzyme is similar to other peroxidases, such as versatile peroxidase or manganese peroxidase, which showed the capability to degrade the lignin of the lignocellulosic crop residues (Ravichandran *et al.* 2019). The *LsLiP* has also been shown that plays an important role in enhancing the decolorization process of dye as evaluated for six different dyes, including azo and anthraquinone. The optimum incubation time required for dye decoloration remarkably depends on the type and chemical structure of dyes (Sudiana *et al.* 2018). When the decolorization of different dyes was tested, purified *LsLiP* showed a higher decolorization rate for anthraquinone dyes than for azo dyes (Fig. 7a). A high decolorization rate was revealed for the anthraquinone dyes, such as NY3 92%, NY5 70.5%, and PB 73.5%, while azo dyes such as NY1, IN13, and NY7 up to 34.5%, 61%, and 56%, within 48 h, respectively. Azo dyes have a complex chemical structure, so they are more difficult to degrade than anthraquinone dyes. Structural bonding in dyes can affect the ability of the LiP enzyme in dye decolorization. Azo dyes have a strong bond between the chromophore

group and auxochrome groups, so they are hard to be biologically decolorized by LiP enzymes, whereas anthraquinone dyes have a weak conjugate bond system. When the LiP enzyme attacks the anthraquinone dyes, electrons from the enzyme are transferred to a structure that has conjugate bonds. This transfer changes the configuration of the dye structure and reduces the color intensity. Taken together, the results of the present study indicate that *LsLiP* from white-rot fungus *L. squarrosulus* MPN12 is involved in the color removal of synthetic dyes and has potential in the biodegradation of lignin (lignocellulosic biomass). However, for future industrial applications, to improve the degradation efficiency of dyes and lignin, LiP should be combined with other ligninolytic enzymes such as (laccase, manganese peroxidase, versatile peroxidases), which are also high redox potential enzymes from white-rot fungi (Yadav and Yadav 2015).

CONCLUSIONS

1. This study is the first to report the secretion of lignin peroxidase (LiP) by the newly isolated *Lentinus squarrosulus* MPN12 of the white-rot fungus.
2. The anion exchange and gel filtration column chromatography techniques were used to purify LiP from *L. squarrosulus* MPN12 (*LsLiP*) up to 47.1-fold. The biochemical properties of *LsLiP* showed that the optimum pH was 5.0, and stability was at the acidic pH range. The optimum temperature was 35 °C for *LsLiP* and the activity was maintained at a moderate temperature (25 to 30 °C). The purified lignin peroxidase from *L. squarrosulus* MPN12 (*LsLiP*) had high specific activity against 2,4-dichlorophenol (DCP) and veratryl alcohol.
3. *LsLiP* from white-rot fungus *L. squarrosulus* MPN12 is involved in the decolorization of synthetic dyes and has potential for the biodegradation of lignin.

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Authors' Contributions

Vu Dinh Giap and Do Huu Nghi managed the project design and funding acquisition. Do Huu Nghi, Dang Thu Quynh, and Le Huu Cuong managed the experimental performance, data analysis, and manuscript preparation. Vu Dinh Giap and Dang Thu Quynh participated in the data analysis and manuscript revision. Dang Thu Quynh joined in the experimental design for optimization.

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