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Purification and characterization of lignin peroxidase from white-rot fungi *Pleurotus pulmonarius* CPG6 and its application in decolorization of synthetic textile dyes

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From the biotechnological point of view, enzymes are powerful tools that help sustain a clean environment in several ways. The enzymatic biodegradation of synthetic dyes is a promising goal since it reduces pollution caused by textile dyeing factory wastewater. Lignin peroxidase (EC 1.11.1.14, LiP) has high redox potential; thus, it is great for application in various industrial fields (e.g., paper- waste treatment and textile dyeing wastewater treatment). In the present study, a LiP from an isolated strain *Pleurotus pulmonarius* CPG6 (*Ppu*LiP) was successfully purified with a specific activity of 6.59 U mg^{-1}. The enzyme was purified by using three-step column chromatography procedures including DEAE, Sephadex G-75, and HiTrap™ Q FF columns with 17.8-fold purity. The enzyme with a molecular weight of 40 kDa exhibited enhanced pH stability in the acidic range. The activity retention was over 75% at a pH of 3.0 for more than 6 hours. Purified *Ppu*LiP was able to oxidize a variety of substrates including veratryl alcohol, 2,4-DCP, n-propanol, and guaiacol. The effect of metal ions on *Ppu*LiP activity was analyzed. The study will provide a ground to decolorize dyes from various groups of *Ppu*LiP. Purified *Ppu*LiP could decolorize 35% Acid blue 25 (AB25), 50% Acid red 129 (AB129), 72% Acid blue 62 (NY3), 85% Acid blue 113 (AB113), 55% Remazol Brilliant blue R (RBBR), and 100% Reactive red 120 (RR120) for 12 hours. Most of the dyes were decolorized, but the heat-denatured enzyme used as negative control obviously did not decolorize the tested dyes. These results indicate that the *Ppu*LiP has potential application in enzyme-based decolorization of synthetic dyes.

Key Words: Decolorization; lignin peroxidase; *Pleurotus pulmonarius*; textile dyes

Introduction

Dyes are drained into the environment as a result of industrial wastewaters, textile, and dyestuff processing. It is estimated that about 10-15% of dyes are released into the wastewater during the dyeing process, which has created a huge pollution problem (Robinson et al., 2001; Chen et al., 2008). The wastewater should be treated before being released into the natural environment. The physical or chemical methods are used due to completion of the process in a short time, but these methods are often very expensive and accumulate a large amount of sludge, which further creates a disposal problem or produces large amounts of toxic iodine (Alam et al., 2009). Secondary pollution is also a problem due to the use of excessive chemicals or a long time process completion. The biological method is more appropriate and widely used due to being more cost-effective and environmentally friendly, especially since the method does not produce large quantities of sludge (Chen et al., 2010). Microbiological decol-

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orization can occur by enzymatic degradation, biosorption, or both of them, but its effectiveness depends on the activities of microbes (Raphael et al., 2018).

The studies showed that bacteria can be used for dye decolorization and degradation (Parshetti et al., 2012; Vignali et al., 2018). However, Bacteria are unable to degrade the dyes efficiently due to the larger size of dyes. The white-rot fungi can produce ligninolytic enzymes (lignin peroxidase, laccase, and manganese peroxidase (Kalyani et al., 2011; Kumar et al., 2020; Sudiana et al., 2018). The enzymes have a wide range of applications and can be used for the removal of dyes from industrial, wastewater, or bio-bleaching effluents (De Souza Silva et al., 2005; Ferreira et al., 2007; Huang et al., 2003). In nature, the oxidative enzymes, especially lignin peroxidase can oxidize both phenolic and non-phenolic lignin-related compounds, resulting in the aromatic ring opening, phenolic oxidation, and the cleavage of the C_{α}-C_{β} bond. Therefore, Lignin peroxidase has great potential for application in various industrial processes and environmental pollutants.

Lignin peroxidase (LiP, EC 1.11.1.14) belong to the family of oxidoreductases, is an extracellular hemeprotein that is H_2O_2-dependent, with an unusually high redox potential. It is capable of oxidizing a variety of reducing substrates, including non-phenolic and phenolic lignin aromatic complex compounds. Numerous LiP have been isolated and characterized from fungal sources including white-rot fungi, such as *Phaenerochaete chrysosporium* (Verma et al., 2002), *Tremetes versicolor* (Jönsson et al., 1994), *Ceriporiopsis subvermispora* (Fernández-Fueyo et al., 2012), brown-rot fungi like *Coniophora puteana* (Irbe et al., 2014), *Laetiporus sulphures* (Mtui et al., 2008).

The current study was designed to evaluate the potential of lignin peroxidase enzymes in the decolorization of various dyes. We purified and characterized a *Ppu*LiP from white-rot fungi *Pleurotus pulmonarius* CPG6, which was isolated from the Cuc Phuong National Park, Viet Nam.

Materials and Methods

Fungal isolation and culture conditions. The fungus was collected from decayed wood logs in the Cuc Phuong National Park, Viet Nam and cultured on 2% malt extract agar supplemented with antibiotics (0.005% streptomycin, penicillin) for 7 days at 30ºC. Then the fungus was cultivated on potato dextrose agar (PDA) and multiplied regularly. The pure culture was kept on PDA, 25% glycerol and stored at -80ºC for further study.

For the production of lignin peroxidase, the fungal strain was grown in a medium containing 10 g L^{-1} glucose, 1.32 g L^{-1} ammonium tartrate, 0.2 g L^{-1} KH_2PO_4, 50 mg L^{-1} $MgSO_4.7H_2O$, 10 mg L^{-1} $CaCl_2$, 10 μg thiamine, and 1 mL L^{-1} trace element solution per liter (3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1 g NaCl, 100 mg $FeSO_4 \cdot 7H_2O$, 80 mg $CaCl_2$, 180 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg H_3BO_3). The pH of the solution was adjusted to 3.5 with dimethyl succinate. The 20 mL growth media contained about 0.5 g wet rice straw in 100 mL culture flasks. The medium was inoculated with mycelia in sterilized condition at 30ºC in an incubator.

Molecular identification and phylogenetic analysis. Fungus was identified by the amplification of the ITS1-5.8S-ITS2 region from the genome of the white-rot fungi *P. pulmonarius*. The ITS1–5.8S–ITS2 region was amplificated using a polymerase chain reaction (PCR) with a forward primer ITS1 (White et al., 1990): 5′-TCCGTAGGTGAACCTGCGG-3′ and a reverse primer ITS4: 5′-TCCTCCGCTTATTGATATTGC-3′. The following cycling conditions were maintained; 3 min initial denaturation step at 95ºC; 35 cycles of 45 min at 94ºC, 45 min at 55ºC, 1 min at 72ºC; and 10 min extension at 72ºC. The amplification of the products was verified by electrophoresis using a 1.5% agarose gel and purified by using QIAquick Gel Extraction Kit (QIAGEN, Germany). Next, amplicons were sequenced in ABI Prism 3100 genetic analyzer (Applied Biosystems, USA). The sequences obtained were analyzed in the ChromasPro1.7.6 (Technelysium, Australia) software and compared with GenBank databases using the NCBI BLAST tool (Johnson et al., 2008).

Phylogenetic analysis: Phylogenetic tree was performed by utilizing the MEGA® v6.0 software (Tamura et al., 2013). The alignment was carried out using Clustal W (Thompson et al., 1997), and the evolutionary history was inferred using the maximum likelihood method (ML) (Harris et al., 1998) based on Kimura's two-parameter model (Kimura et al., 1980) with 1000 bootstrap replicates. Treview software is used to edit evolutionary tree images (Page et al., 1996).

Enzyme activity assay. Lignin peroxidase activity was determined by monitoring the veratraldehyde that formed in a reaction mixture of 1 mL containing 100 mM veratryl alcohol (Sigma), 250 mM sodium tartrate buffer, pH 3.0, 10 mM H_2O_2, and an appropriate amount of *Ppu*LiP (Tien et al., 1984). The enzyme assay was measured at 310 nm for 1 min at 30ºC. One unit of LiP activity was defined as 1 μmol veratraldehyde formed/min (molar extinction coefficient, E_{max} = 9300 M^{-1} cm^{-1}).

Enzyme purification. The 10 days old cultured media were harvested at the maximum activity of lignin peroxidase. The proteins were extracted with the distilled water, then the mycelium and straw particles were removed by centrifugation (10,000× g for 10 min) and filtration (filter GF6; Whatman, UK). The clear supernatant was harvested and concentrated by in a tangential flow ultrafltration system at 11°C (10 kDa cut-of; Sartorius, Germany). The culture fluid was subsequently applied to a HiTrap™ DEAE cellulose fast flow column equilibrated with buffer A (10 mM sodium acetate, pH 4.5). The adsorbed protein was washed with the same buffer and eluted by NaCl gradient (0-1.5 M) at a flow rate of 2 mL min^{-1}. Next, the active fractions were pooled and applied to Sephadex G-75 gel filtration column in buffer B (10 mM sodium acetate, pH 4.5, 50 mM NaCl) at a flow rate of 1 mL min^{-1}. The final purification step was occurred on a HiTrap™ Q FF column equilibrated with buffer B. Proteins were eluted by a linear gradient of 0.25-1.0 M NaCl in buffer B at a flow rate of 0.5 mL min^{-1}. Next, Active fractions were pooled and desalted in buffer A using a 5 mL-Amicon Ultra centrifugal filer (10 kDa). The concentrated protein was stored at -80ºC and was used for further studies.

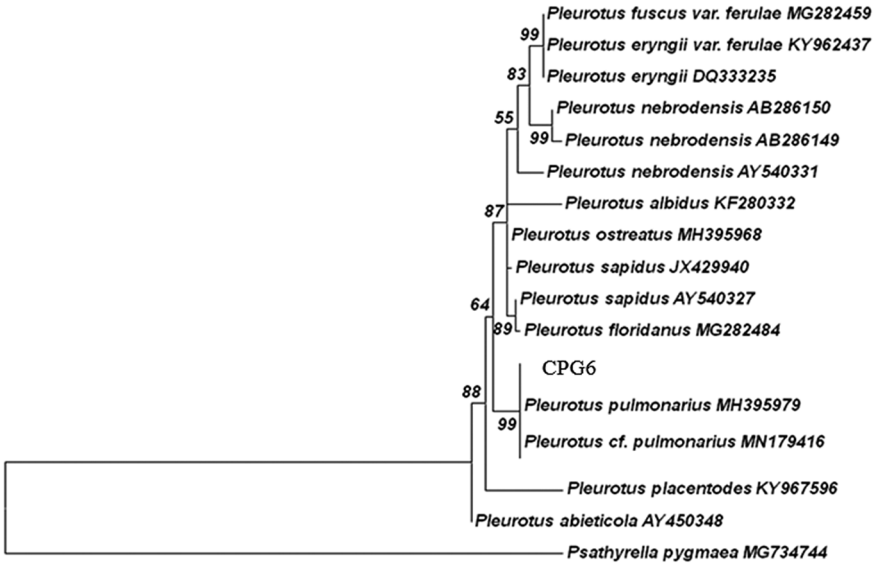

Fig. 1. Phylogeny tree of fungi (ITS region).

Phylogeny tree was performed based on ML method using partial rDNA-ITS sequence of the CPG6 fungal strain. *Psathyrella pygmaea* MG734744 was used as an outgroup taxon. The names of all fungal species in the phylogenetic tree were followed by the Accession Numbers.

All purification steps were carried out using an ÄKTA Pure system (GE Healthcare, Danderyd, SWE) with a detector operating at 280 nm at 4ºC.

Enzyme characterization. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using method of Weber and Osborn (Weber et al., 1969). The separating gel was 12% acrylamide in 0.375 M Tris-HCl buffer pH 8.8 and stacking gel 5% acrylamide in 0.063 M Tris-HCl buffer 6.8. Protein bands was stained with 0.1% Coomassie brilliant blue R-250 (w/v) in acetic acid/methanol/water (v/v/v) (1:4:5) for 1 hour at room temperature, followed by destaining in acetic acid/methanol/water (v/v/v) (1:1:8) for overnight at room temperature.

The optimum pH of purified *Ppu*LiP was measured in the pH range 1.0-7.0 (0.1 M sodium-tartrate buffer, pH 1.0-3.0; 0.1 M acetate-acetic acid buffer, pH 4.0-5.0; sodium-phosphate buffer, pH 6.0-7.0). The temperature optima for the purified *Ppu*LiP were determined by measuring the enzyme activity at 20-45ºC, as described above in the enzyme activities.

The stability of pH of *Ppu*LiP was determined by measuring the residual activity level, as described in the enzyme activity assay. The *Ppu*LiP enzyme was incubated at various pH values in 0.1 M sodium-tartrate buffer, pH 3.0; 0.1 M acetate-acetic acid buffer, pH 5.0; sodium-phosphate buffer, pH 7.0 for 6 hours at an interval time of 1 hour. The thermal stability was determined at various temperatures (20, 30, 40, 60ºC) for up to 2 hours at an interval time of 20 min before enzyme incubation was considered 100%.

To elucidate the effects of different metal ions on the activity of *Ppu*LiP, 1 mM (final concentrations) of KCl, NaCl, $CaCl_2$, $MnSO_4$, $ZnCl_2$, $HgCl_2$, $Cu(SO_4)_2$, $FeCl_2$, $MgCl_2$, and EDTA was added into the reaction mixture separately, and *Ppu*LiP activity was measured at 30ºC. The activity was determined using veratryl alcohol as a substrate.

Enzyme kinetics. The Michaelis-Menten constant (K_m) and catalytic rate constant (k_{cat}) were determined by measuring the activity of *Ppu*LiP for various LiP substrates (Tab. 2). The K_m and k_{cat} parameters were calculated by applying the Lineweaver-Burk plot. Substrate concentrations were varied from 0 to 150 μM at optimum pH and temperatures. Kinetics constant for Veratryl alcohol, n-propanol, and guaicol were measured at 310 nm, while for 2,4-dichlorophenol (2,4-DCP) were assayed at 510 nm. All experiments were repeated three times.

Decolorization assay. The decolorization of 6 different dyes by purified *Ppu*Lip was measured after 12 hours incubation at the λmax of each dye (Tab. 3). The reaction mixture containing 250 mM sodium tartrate buffer, pH 3.0, 50 mM H_2O_2, an appropriate amount of *Ppu*LiP and dye with a final concentration of 150 mg/L. Assay was measured at 30ºC and the reaction was initiated with the addition of the enzyme. All experiments were repeated three times. The percentage of decolorization was calculated using equation (1)

$$\text{Decolorization}(\%)\ \frac{(Ai\text{-}At)}{Ai}\times 100 \quad (1)$$

Where *Ai* is initial absorbances of the mixture and *At* is the final absorbances of the mixture.

Results and Discussion

Identification of isolated fungal strain with high LiP activity

These screened isolates were identified on a morphological basis and then reconfirmed by PCR, amplifying ITS sequences with primers (ITS1/ITS4). A sequence of 703 nucleotides in length was obtained and deposited in GenBank nucleotides database (http://www.ncbi.nlm.nih.gov/genbank/) with the accession number MZ097345. The

sequence obtained was then compared with those available in GenBank database by applying the NCBI BLAST tool. The fungal isolates were found to be closely related to *Pleurotus pulmonarius* MH395979 species (100%). Based on this genetic identification, the strain CPG6 was authenticated as *Pleurotus pulmonarius* (*Pleurotaceae*, 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.

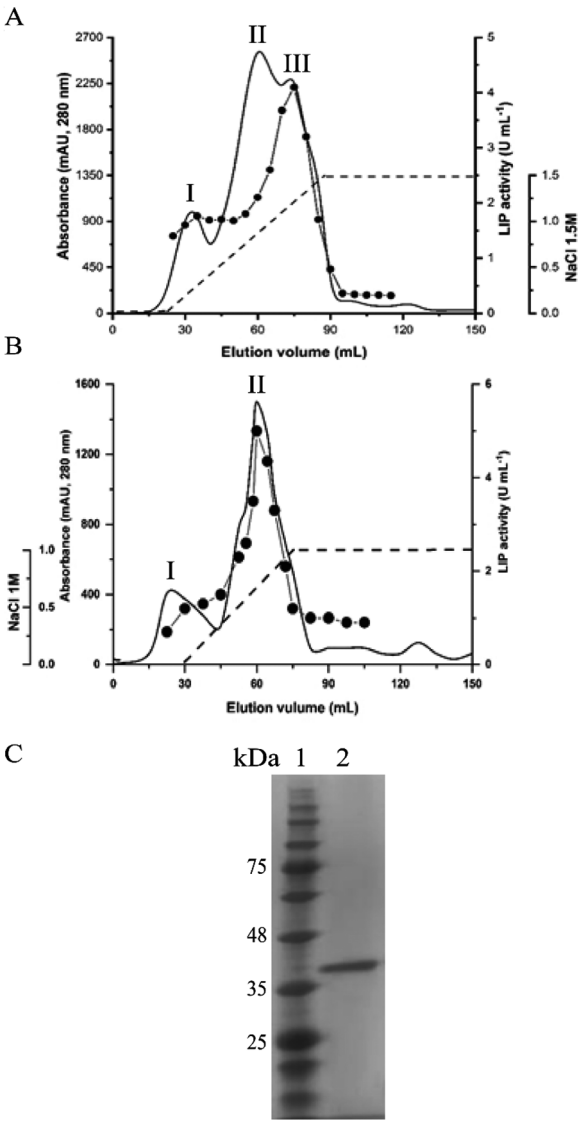

Fig. 2. Elution profles of the *Ppu*LiP.

Purification of *Ppu*LiP by anion-exchange chromatography on a DEAE cellulose column (A), HiTrap^{TM} Q column (B): absorbance at 280 nm (solid line), LiP activity (black circles), and NaCl gradient (dashed line). and SDS-PAGE gel after protein purification (Lane 1: marker, lane 2: eluted fraction from HiTrap^{TM} Q column) (C).

Lignin peroxidase purification

To characterize the LiP from white-rot fungi *P. pulmonarius* CPG6, we performed protein purification by three-step column chromatography procedures including DEAE, Sephadex G-75, and HiTrap^{TM} QFF. First, the culture fluid obtained was filtered and concentrated by filtration in the method section, after which the protein was applied to weak anion exchange chromatography on the DEAE-Cellulose column. The separation by the DEAE column was the most effective step in the purification procedure, the specific activity of *Ppu*LiP increased from 0.37 U mg^{-1} to 3.64 U mg^{-1} in the crude protein after passage through on a DEAE column (Tab. 1). Fig. 2A showed the elution pattern of *Ppu*LiP on DEAE column, and obtained three well-separated peaks (I, II, and III). Peak III was high having high *Ppu*LiP activity (4.2 U mL^{-1}), while in peaks I and II *Ppu*LiP activity was 1.9 and 2.2 U mL^{-1}, respectively. The protein of peak III was collected and desalted in a Sephadex G-75 gel filtration column. Proteins were subsequently loaded into a HiTrap^{TM} QFF. The elution pattern of *Ppu*LiP was obtained and two peaks (I and II) well were separated. Peak II has a high protein concentration and lignin peroxidase activity (Fig. 2B). The purified protein appeared on an SDS-PAGE gel as a single band with a mass of 40 kDa (Fig. 2C), a yield of 59% and a purification fold of 17.8% (Tab. 1). The molecular weight of *Ppu*LiP is consistent with the range molecular weight of lignin peroxidase of fungi from the earlier report (38-50 kDa) (Sugiura et al., 2003; Vandana et al., 2019; Yadav et al., 2009).

Effect of the pH and temperature on the PpuLiP activity

The effect of pH and temperature on *Ppu*LiP activity was determined using veratryl alcohol as a substrate. The optimum pH of *Ppu*LiP was measured between pH 1.0-7.0, while the highest activity was observed at pH 3.0 (Fig. 3A). The optimum pH range of *Ppu*LiP was different from other LiPs. Most of LiPs from fungi showed higher activity at the pH range of 5.0 to 6.0, such as LiP from *Phanerochaete chrysosporium* activity was optimum at a pH of 5.5 (Vandana et al., 2019). Asgher study showed that LiP from *Schizophyllum commune* activity was optimum at pH 5.0 (Asgher et al., 2016). In our study, the purified LiP exhib-

Table 1. Purification summary of *Ppu*LiP.

Purification steps [a)]	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Culture fluid	950	360	0.37	100	1.0
DEAE Cellulose	74	270	3.64	75	9.8
Sephadex G-75	53	251	4.73	68	12.7
HiTrap^{TM} Q FF	32	211	6.59	59	17.8

[a)] Protein purification by three-step column chromatography procedures including DEAE, Sephadex G75, and HiTrap^{TM} QFF.

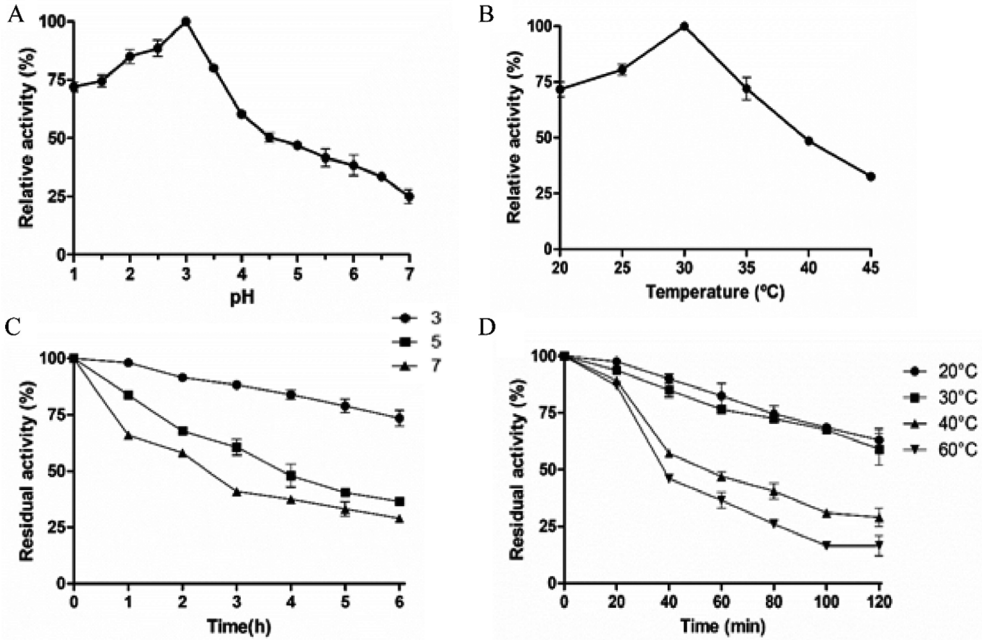

Fig. 3. Effect of the pH and temperature on the *Ppu*LiP activity.

A. Optimum pH. *Ppu*LiP was determined at various (pH 1.0-7.0), as described in the Materials and method section. B. Optimum temperature. *Ppu*LiP activity was measured at pH 3.0 with different temperatures (20-45ºC). C. pH Stability. pH stability of *Ppu*LiP was determined by measuring the residual activity at 30ºC after incubation of the enzyme at pH 3; 5 and 7 for indicated times. D. Thermal stability. Thermal stability of *Ppu*LiP was determined by measuring the residual activity at pH 3.0 after incubation of the enzyme at 20-60ºC for the indicated times. Enzyme activity before incubation was 100%. Each value represents the mean ± SD of three measurements.

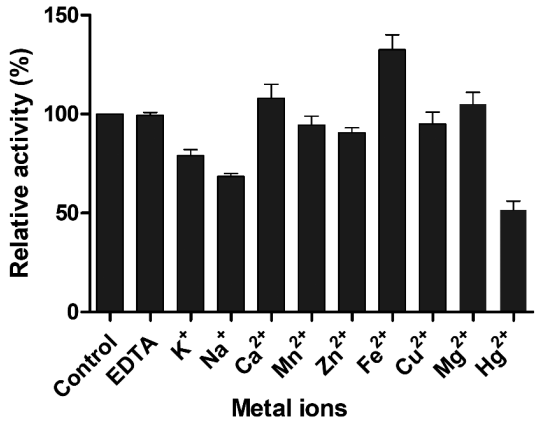

Fig. 4. Effect of metal ions on activity of *Ppu*LiP.

The effects of different metal ions on the activity of *Ppu*LiP, 1 mM (final concentrations) of K^+, Na^+, Ca^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and EDTA was added into the reaction mixture separately, and *Ppu*LiP activity was measured at 30ºC. The activity was determined using veratryl alcohol as a substrate. Each value represents the mean ± SD of three measurements.

ited enhanced pH stability in the acidic range. The activity retention was over 75% at a pH of 3.0 for more than 6 hours (Fig. 3C). However, at pH 5.0 and 7.0, the activities were decreased to 38% and 29% of the initial activities, respectively. *Ppu*LiP was similar to the purified LiP from *P. chrysosporium* (Vandana et al., 2019) and *Penicillium decumbens* (Yang et al., 2005), which showed stability in the acidic environment (pH 4.0) with 75% and 70.5% of the initial activity, respectively.

Most of the LiPs from other white-rot fungi showed an optimal temperature range of 35ºC to 60ºC. LiP from *P. decumbens* and *Humicola grisea* showed optimum activity at 45ºC and 60ºC, respectively (Yang et al., 2005; Moubasher, et al., 2017). In our study, the optimal temperature of *Ppu*LiP was measured between 20-45ºC (Fig. 3B). *Ppu*LiP exhibited optimum activity at 30ºC. The purified enzyme showed a higher thermostability at moderate temperature (20-30ºC), while the activity down rapidly at high temperature (Fig. 3D). The activity loss was 25% and 20% at 40ºC and 60ºC, respectively during 120 min of incubation. These results show that the LiP from *P. pulmonarius* was not stable at high temperatures. Our study is not in line with previous studies, such as LiP from *P. decumbens* and *P. chrysosporium* were thermostable at 60ºC and 65ºC, respectively (Yang et al., 2005; Vandana et al., 2019).

Effect of metal ions on the PpuLiP activity

The effect of a few metal ions at 1 mM was determined with veratryl alcohol, the main substrate at room temperature. *Ppu*LiP activity was slightly enhanced by adding Ca^{2+}, Fe^{2+} and Mg^{2+} (112%, 125%, and 110%, respectively). Louie also reported that Fe^{2+} can coordinate to the active sites leading to activation of the enzyme (Louie et al., 1999). The activity was inhibited by K^+, Na^+, and Hg^{2+} (75%, 62%, and 48%, respectively), while the addition of Mn^{2+}, Zn^{2+}, Cu^{2+} or ethylenediaminetetraacetic acid (EDTA) had no effect on *Ppu*LiP activity (Fig. 4). In industrial applications, the activity of peroxidase in the presence

Table 2. Kinetic Parameters of *Ppu*LiP.

Substrate	K_m, μM [a]	k_{cat}, s^{-1} [b]	k_{cat}/K_m, $\mu M^{-1}.s^{-1}$ [c]
Veratryl alcohol	25 ± 0.91	3.4 ± 0.07	0.136
2,4-DCP	28.5 ± 1.13	2.8 ± 0.04	0.098
n-propanol	61 ± 2.01	2.1 ± 0.08	0.035
Guaicol	54 ± 1.52	2.3 ± 0.03	0.042

[a] The Michaelis-Menten constant (K_m), [b] catalytic rate constant (k_{cat}), and [c] catalytic efficiencies (k_{cat}/K_m)

Table 3. Dye decolorization of crude and purified *Ppu*LiP.

Compound	λ_{max}, nm [a]	Degradation (%) [b]		Increase (%)
		Crude *Ppu*LiP	Purified *Ppu*LiP	
Acid blue 25 (AB25)	603	15	35	20
Acid red 129 (AB129)	629	35	50	15
Acid blue 62 (NY3)	595	46	72	26
Acid blue 113 (AB113)	543	61	85	24
Remazol Brilliant blue R (RBBR)	592	32	55	23
Reactive red 120 (RR120)	512	70	100	30

[a] The absorbance at the respective wavelengths of the substrate
[b] The decolorization of 6 different dyes by purified *Ppu*Lip was measured after 12 hours incubation at the λmax of each dye. The percentage of decolorization was calculated using equation (1)

of metal ions is an essential property and has been reported previously in numerous studies (Lee et al., 2001; Yuzugullu Karakus et al., 2018).

Enzyme kinetics

Kinetic constants of purified *Ppu*LiP were studied with veratryl alcohol, 2,4-DCP, n-propanol, and guaicol as substrate. The reaction rates were varying at substrate concentration in 250 mM sodium tartrate buffer (pH 3.0). Michaelis-Menten constants (K_m = 25 up to 61 μM) and catalytic efficiencies (k_{cat}/K_m values from 0.035 to 0.136 $\mu M^{-1}.s^{-1}$) could be determined for all tested substrates. The highest affinity (K_m = 25 μM) and catalytic efficiencies (k_{cat}/K_m = 0.136 $\mu M^{-1}.s^{-1}$) was ascertained for veratryl alcohol, which is also thought to be the physiological substrate of *Ppu*LiP. In relation to veratryl alcohol, the catalytic efficiencies of *Ppu*LiP was in descending order i.e. 2,4-DCP > guaiacol > n-propanol (Tab. 2). Lignin peroxidase was able to oxidize a number of phenolic compounds in a wide variety of applications. Among the various substrates tested, 2,4-DCP and veratryl alcohol as the main substrate of LiP have been studied the most (Casciello et al., 2017; Koduri et al., 1994; Ollikka et al., 1993; Romero et al., 2019; Vandana et al., 2019).

The kinetic data in the K_m values using veratryl alcohol as the substrates have been reported previously in numerous studies. The lignin peroxidase of *P. pulmonarius* showed a K_m value of 25 μM, which was 2.4-folds lower than that obtained for *P. sanguineus*, *P. chrysosporium*, and *Aspergillus terreus* (61 μM, 60 μM, and 60 μM, respectively) (Sharma et al., 2011). Thus, *Ppu*LiP showed a significantly higher substrate-binding affinity than the other LiPs. Moreover, the catalytic efficiency (k_{cat}/K_m) value of *Ppu*LiP (0.136 $\mu M^{-1}.s^{-1}$) was 2.1 and 3.8-fold higher than that of LiP from *P. sordida* (0.063$\mu M^{-1}.s^{-1}$) and *P. sanguineus* (0.035 $\mu M^{-1}.s^{-1}$), respectively (Sugiura et al., 2003; Sharma et al., 2011). Taken together, the results suggest that the *Ppu*LiP is not in range with previous studies, the affinity of lignin peroxidase from *P. pulmonarius* CPG6 with veratryl alcohol is higher than that from other fungal strains.

Decolorization of dyes

In this present study, we wanted to characterize the ability of the lignin peroxidase to use different dye compounds as substrates, purified *Ppu*LiP after 12 hours of incubation, decolorized all dyes. However, the heat-denatured enzyme had no activity on the test dyes. Purified LiP from *P. pulmonarius* CPG6 decolorized six different dyes belonging to the azo and anthraquinone group at acidic pH, indicating the enzyme decolorization ability at acidic pH plays a significant role in dye decolorization. Tab. 3 shows the decolorization of dyes during crude and purified *Ppu*LiP after incubating. Purified *Ppu*LiP completely (100%) decolorized RR120 and with a 30% enhancement in comparison to the degradation of the crude enzyme. The 85% degradation obtained with AB113 and 72% with NY3, also reflected a 24% and 26% enhancement, respectively, in comparison to the decolorization of the crude enzyme. Color removal rates were 55% and 50% for RBBR and AB129, respectively. AB25 was difficult to be decolorized with both crude (15%) and purified *Ppu*LiP (35%).

Decolorization of synthetic dyes has also been reported and corroborated by previous observations (Ghodake et al., 2009; Ollikka et al., 1993; Verma et al., 2002; Park et al., 2004). Notably, the purified LiPs decolorized a wide range of dyes, including azo, triphenylmethane, heterocyclic, polymeric, and metal complexes at neutral pH. Parshetti et al. showed that the purified LiP from *Kocuria rosea* decolorized of dyes with high efficiency at pH 7.0, such as methyl orange (100%), orange HE2R (90%), or

reactive blue 25 (100%) (Parshetti et al., 2012). The purified LiP from *P. chrysosporium* also showed a 2-fold higher decolorization rate at neutral pH (5.0-6.0) than at acidic pH (3.0) (Verma et al., 2002). Interestingly, in this study we showed purified LiP from *P. pulmonarius* CPG6 has the best decolorization ability at acidic pH, indicating the efficiency of decolorization by the enzyme related to the pH dependence.

Conclusions

The anion exchange and gel filtration column chromatography technique were used to purify LiP enzyme from *P. pulmonarius* CPG6 up to 17.8-fold. The molecular weight on SDS-PAGE of *Ppu*LiP was 40 kDa. Biochemical properties of *Ppu*LiP showed that the optimum pH was 3.0 and stability at the acidic pH range. The optimum temperature was 30ºC for *Ppu*LiP and the activity was maintained at moderate temperature (25-30ºC). The purified lignin peroxidase from *P. pulmonarius* CPG6 (*Ppu*LiP) has high specific activity with veratryl alcohol as a substrate. The study suggests that *Ppu*LiP is suitable for the decolorization of synthetic dyes.

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