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Full Length Research Paper

Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation

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Laccase production by the white rot fungus, *Ganoderma lucidum*, was investigated using submerged fermentation, and the laccase was purified and characterized. The effects of variations in the media nutrient composition or culture conditions and characteristics of purified laccase were provided for large scale production or industrial uses of the laccase. Low concentrations of glucose and yeast extract stimulated *G. lucidum* laccase production. The addition of lignocellulosic substrates to the media stimulated laccase production: pomelo peel induced the highest laccase activity of 11842.13 U/L and banana peel reduced laccase production, compared to control media containing wheat bran. Native PAGE and SDS PAGE demonstrated the presence of a single isoform with a molecular weight of 68 kDa. The optimum pH and temperature were 3.0 and 60°C, respectively. *G. lucidum* laccase is thermostable, with a residual activity of 46% after 80 min at 60°C. The Michaelis-Menten constant (K_m) and catalytic constant (K_{cat}) values for *G. lucidum* laccase using ABTS (2,2'-azino-di-[3-ethyl-benzothiazolin-sulphonate]) as a substrate were 0.114 mM and 74.63 S^{-1}, respectively, with a specificity constant (K_{cat} / K_m) of 654.65 S^{-1} mM^{-1}. The thermostable properties of the laccase produced by fermentation of *G. lucidum* in submerged culture had potential for industrial and biotechnology applications.

Key words: Laccase, *Ganoderma lucidum*, submerged fermentation, pomelo peel.

INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing enzyme which is widely distributed within plants, insects and fungi. Laccase catalyzes the removal of electrons from a variety of organic substrates, including phenols, methoxyphenols, aromatic amines, while reducing molecular oxygen to water as a final electron acceptor. Unlike lignin peroxidase and manganese peroxidase, laccase is capable of catalyzing the oxidation of organic compounds in the absence of H_2O_2 or Mn^{2+} (Leonowicz et al., 2001), allowing laccase to be effectively applied within several industrial processes, including bleaching in the paper industry (Oudia et al., 2008), decolorization of textile dyes (Murugesan, 2007, 2009a; Mazmanci et al., 2009; Moturi and Charya, 2010), removal of herbicides from cereal crops (Coelho et al., 2010) and the biodegradation and bioconversion of food and agricultural waste (Sánchez, 2009; Haddadin et al., 2002; Strong and Burgess, 2008).

White-rot fungi (WRF) are laccase producing species of significant interest, as they secrete laccase to degrade the lignin component of wood to expose cellulose (Paterson, 2007). The WRF species *Trametes versicolor* (Lorenzo et al., 2002), *Pleurotus ostreatus* (Liu et al.,

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2009; Hou et al., 2004), *Agaricus blazei* (Ullrich et al., 2005), *Clitocybe maxima* (Zhang et al., 2010), *Pleurotus eryngii* (Wang and Ng, 2006a) and *Pycnoporus sanguine* (Vite-Vallejo et al., 2009) have been studied in detail for many years and are candi-dates for industrial application. *Ganoderma lucidum* is an important medicinal mushroom in China which has recently been employed in the solid state fermentation of laccase.

Laccase synthesis is known to be influenced by the culture conditions, including variations in the types and concentration of nutrients available. Investigation of the effects of different nutrients in the culture media on laccase synthesis is necessary for large scale laccase production processes. Some lignocellulosic substrates released by agricultural, food and forestry processes have been used in laccase fermentation (Haddadin et al., 2002; Elissetche et al., 2007; Songulashvili et al., 2007). These materials can provide essential nutrients for fungal growth (Grohmann et al., 1995) and also contain components which stimulate laccase production (Lorenzo et al., 2002). However, the effect of altered media compositions in submerged laccase fermentation has not been determined. Additionally, the effect of lignocellulosic substrates on the fermentative production of laccase by *G. lucidum* has been poorly investigated.

In this work, *G. lucidum* laccase production was investigated using submerged fermentation and the laccase was purified and characterized. The stimulatory effects of lignocellulosic substrates on laccase production and the thermostability of *G. lucidum* laccase have potential applications within industry and biotechnology.

MATERIALS AND METHODS

Fungal strain and culture conditions

The white-rot fungus *Ganoderma lucidum* was maintained on potato dextrose agar (PDA) slants. The slants were inoculated and incubated at 30°C for 16 days and then stored at 4°C. For laccase production, *G. lucidum* was cultivated in 150 ml fermentation medium in 500 ml Erlenmeyer flasks at 30°C with shaking at 150 rpm. Fermentation medium was composed of 20 g glucose, 10 g wheat bran, 2 g $MgSO_4 \cdot 7H_2O$ and 3 g KH_2PO_4 per liter. Seed cultures were cultivated in fermentation medium at 30°C and 150 rpm for 14 days.

Laccase activity assay

Laccase (EC 1.10.3.2) activity was measured spectrophotometrically determined by measuring the increase in absorbance at 420 nm, 30°C using 1 mM ABTS (2,2'-azino-di-[3-ethyl-benzothiazolin-sulphonate]) as the substrate (Hou et al., 2004). The 1 ml assay mixture contained 880 µl 100 mM sodium acetate buffer (pH 4.5), 100 µl ABTS stock (final assay concentration 1 mM) and 20 µl appropriately diluted crude culture broth or purified enzyme. The enzyme activity was calculated using the molar extinction coefficient of oxidized ABTS ($\varepsilon_{420} = 3.6\times 10^4\ M^{-1}cm^{-1}$) with one unit of activity defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute.

Carbon and nitrogen sources and lignocellulose substrate experiments

To study the effect of different carbon sources on laccase production, *G. lucidum* was grown in fermentation media containing 20 g/L or 80 g/L lactose, sucrose, maltose or starch, instead of glucose. The control media for this experiment contained no sugar. Fermentation media containing 20 g/L glucose was used to study the effect of different nitrogen sources on laccase production. Then, inorganic or organic nitrogen sources were added to the fermentation media at a final concentration of 10 g/L. The control media was fermentation media.

Lignocellulosic substrates were obtained from pomelo, orange, mandarin orange or banana fruit by drying the peels at 60°C and milling each to a powder. To investigate the effects of the lignocellulosic substrates on laccase production, the different materials were added to the fermentation media at a final concentration of 10 g/L, instead of wheat bran. The control media was fermentation medium with 10 g/L yeast extract.

Each experiment was performed in trireplicate. The results depicted were the averages of three replicates and the error bars indicated standard deviations.

Purification of laccase

The crude laccase was purified from culture broth containing pomelo peel as a lignocellulosic substrate using two $(NH_4)_2SO_4$ precipitation steps. 40% saturated solution of $(NH_4)_2SO_4$ was used to remove hybrid proteins from the fermentation broth, and then 70% saturated $(NH_4)_2SO_4$ was used to precipitate laccase from the supernatant obtained in the first step. The protein precipitate was dissolved in 50 mM (pH 3.0) sodium acetate buffer, dialyzed in the same solution to remove $(NH_4)_2SO_4$ and the desalted enzyme solution was applied to a DEAE FF 16/10 column pre-equilibrated with pH 7.0 sodium phosphate buffer solution (buffer A). The column was washed with buffer A and the absorbed proteins were eluted using a linear gradient of 0 to 1.0 mol/L NaCl in buffer A over 40 min at flow rate of 5 ml/min. The active fractions were pooled and dialyzed against buffer A, concentrated to 2 ml by lyophilization and passed through a 2.5 × 100 cm Sephacryl S-100 HR column equilibrated with buffer A. The column was washed at 1 ml/min with buffer A and the active fractions were pooled. Finally, the active fractions were concentrated by lyophilization, and the purified laccase was used in the subsequent experiments.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE without SDS were performed in accordance with standard methods (Laemmli, 1970) using 10% resolving gels and 5% stacking gels. Activity staining of laccase was performed by incubating the native PAGE gel in 50 mM sodium acetate buffer solution (pH 4.0) containing 1 mM ABTS for 30 min at room temperature.

Effect of pH and temperature on laccase activity

To determine the optimal pH, laccase activity was measured over a pH range of 1.0 to 7.0 using ABTS as substrate. The pH gradient was obtained by sodium acetate buffer (2.0 to 7.0) and HCl – KCl buffer (1.0). The effect of temperature on laccase activity was examined over a temperature range of 20 to 80°C, by incubating the assay reactions at various temperatures for 10 min

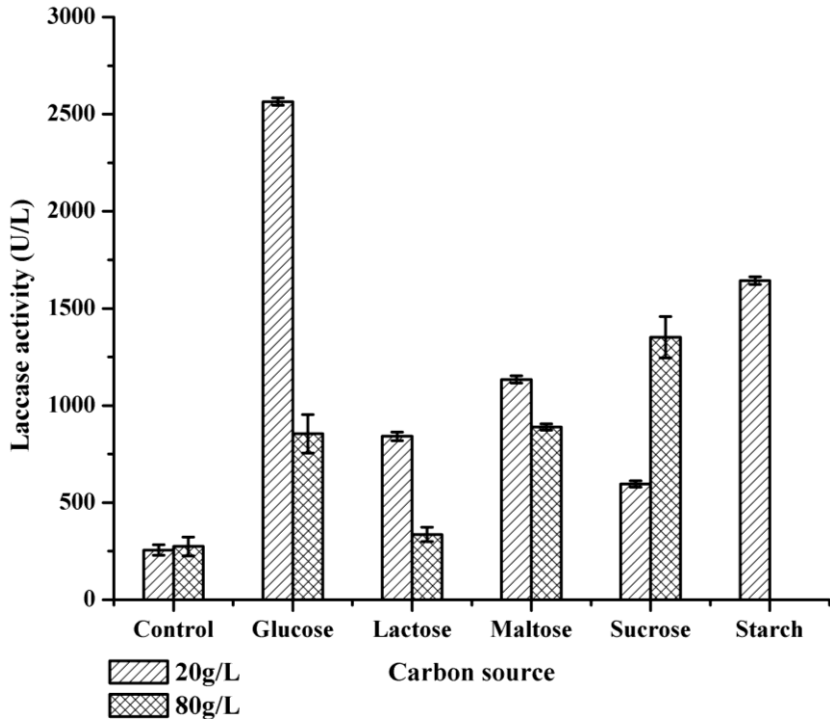

Figure 1. Effect of varied concentrations of different carbon sources on laccase production by *Ganoderma lucidum* in submerged fermentation conditions. The control media was composed of fermentation media without sugar.

before the addition of enzyme and determination of laccase activity using ABTS as substrate.

pH stability and thermostability of laccase

To investigate the effect of pH on enzyme stability, the purified enzyme was incubated at room temperature for 240 min in buffers of various pHs, and then residual laccase activity assay was determined using ABTS as substrate. The thermostability of the enzyme was determined by measuring laccase activity every 10 min for 80 min using ABTS as substrate at optimal pH over a temperature range of 30 to 80°C.

Kinetic properties of laccase

To determine the Michaelis-Menten constant (K_m) and catalytic constants (K_{cat}) of laccase, varying concentrations of ABTS solution were mixed with purified laccase, the standard laccase enzyme assay was performed and the K_m and K_{cat} values were determined using a Lineweaver-Burk plot.

RESULTS

Effect of carbon and nitrogen sources on *G. lucidum* laccase fermentation

Figure 1 shows that, of the five different sugars tested, 20 g/L glucose was the most effective sole carbon source, resulting in the largest increase in laccase production compared to the control media, containing no added sugar. At 20 g/L, glucose stimulated the maximal laccase activity of 2,564.86 U/L. When the sugar concentrations were increased to 80 g/L, laccase production decreased in all of the five carbon sources tested, with the highest activity of 1,351.41 U/L obtained using sucrose as a carbon source.

The effect of the addition of different nitrogen sources to the fermentation media is shown in Figure 2. At 10 g/L, the different organic and inorganic nitrogen sources had varied effects on the production of laccase by *G. lucidum*. Inorganic nitrogen sources, including ammonium sulfate and urea, had an inhibitory effect on laccase production compared to the control media. The presence of yeast extract effectively improved the synthesis of laccase, stimulating a laccase activity of 4,904.38 U/L.

Effect of lignocellulosic substrates on G. lucidum laccase fermentation

The effect of four different lignocellulosic substrates on laccase production was investigated. Pomelo, orange and mandarin orange exerted a stimulatory effect on *G. lucidum* laccase production, compared to the control media. After 7 days culture, the highest laccase activity of 11,842.13 U/L was observed in the pomelo peel

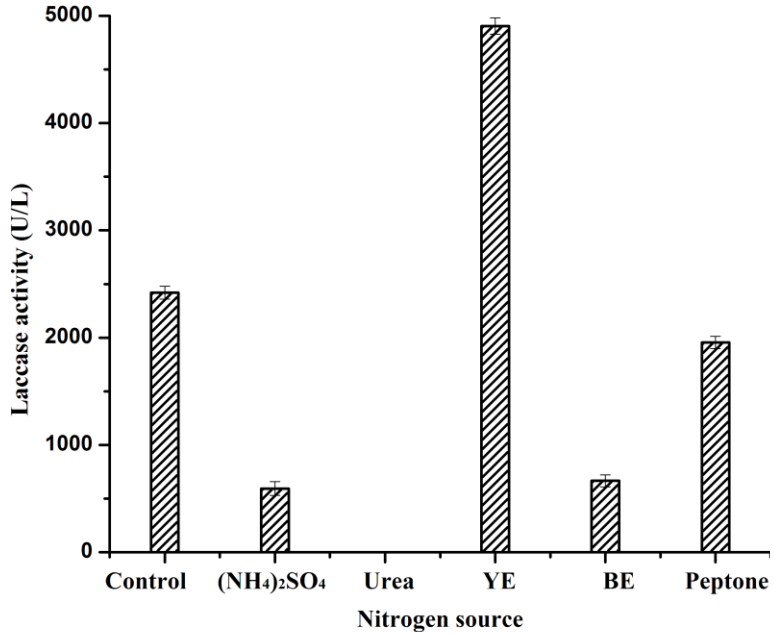

Figure 2. Effect of different nitrogen sources on laccase production by *Ganoderma lucidum* in submerged fermentation. The control media contained wheat bran; YE, yeast extract; BE, beef extract.

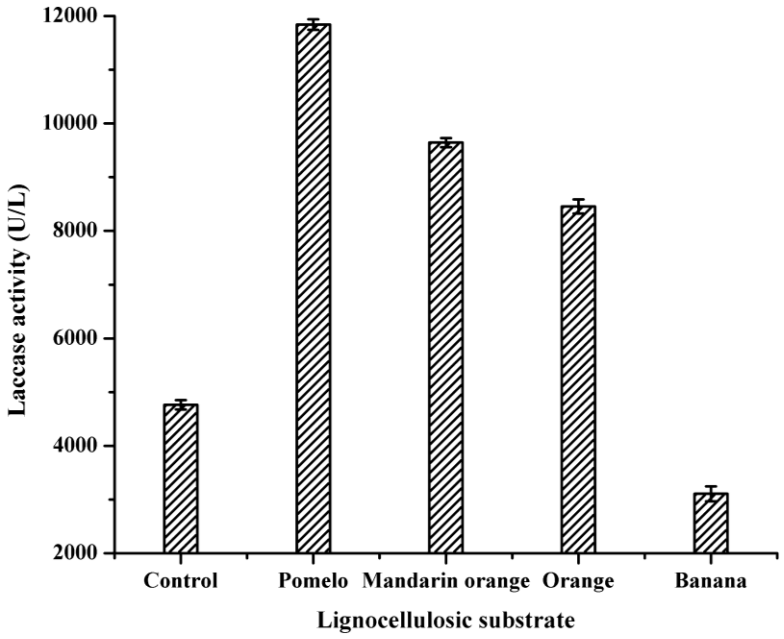

Figure 3. Effect of different lignocellulosic substrates on laccase production by *Ganoderma Lucidum* in submerged fermentation. Control media contained wheat bran instead of 10 g/L lignocellulosic substrate.

media, followed by the media containing orange or mandarin orange peel, which had laccase activities of approximately 9,000 U/L (Figure 3). In contrast, the addition of banana peel to the submerged culture had a negative effect on laccase production, leading to a lower activity than the control media.

Purification and gel electrophoresis

After purification using a Sephacryl S-100 HR column, *G. lucidum* laccase was purified 7.19-fold compared to the crude extract, with a 30.65% yield and specific activity of 39.25 U/mg (Table 1). The purified laccase was analyzed by native-PAGE and SDS-PAGE. The purified protein demonstrated a single band in SDS-PAGE analysis, with a molecular weight of 68 kDa (Figure 4A). One major band was observed after incubating the native-PAGE gel with the laccase substrate ABTS, indicating the presence of a single laccase isoform in the purified enzyme extract (Figure 4B).

Effect of pH and temperature on laccase activity

The effects of pH values ranging from 1.0 to 7.0 on laccase activity were investigated (Figure 5A). Laccase exhibited the highest activity at pH 3.0. Enzyme activity decreased sharply as the pH value increased from 3.0 towards the neutral range, and laccase was almost completely inactivated at pH 7.0.

The influence of temperature on laccase activity was determined at temperatures ranging from 20 to 80°C, and the optimal temperature for laccase was determined to be 60°C (Figure 5B). Enzyme activity declined when the temperature was increased from 60 to 80°C, with 40% of the optimal enzyme activity observed at 80°C.

pH stability and thermo-tolerance of laccase activity

The pH stability of the purified laccase was investigated, by assaying residual laccase activity after incubation of the enzyme for 240 min at pH values ranging from 2.0 to 7.0 (Figure 6A). Laccase enzyme activity was stable at pH 3.0, retaining more than 80% activity after 240 min. In contrast, less than 10% of the laccase activity remained after 240 min incubation at pH 2.0, 6.0 and 7.0.

The thermal stability of laccase was investigated by incubating the enzyme at different temperatures at pH 3.0 and assaying laccase activity every 10 min, for 80 min (Figure 6B). No loss in laccase activity was detected over 80 min at 30, 40 or 50°C. At 60°C, 46% of the residual laccase activity remained after 80 min, indicating that *G. lucidum* laccase is a thermostable enzyme. Less than 10% of laccase activity was retained after 20 min when incubated at 70°C, and complete inactivation occurred after 10 min at 80°C.

Kinetic properties of the laccase enzyme

The kinetic parameters of purified laccase were characterized using the Michaelis constant K_m, catalytic constant K_{cat} and specificity constant (K_{cat} / K_m) using various concentrations of ABTS as a substrate. The Lineweaver-Burk plot (Figure 7) indicated that the K_m and catalytic constant K_{cat} values for purified laccase were 0.114 M and 74.63 S^{-1}, respectively, and the specificity constant (K_{cat} / K_m) was 654.65 S^{-1} mM^{-1}.

DISCUSSION

The major component of the natural resources released by farming and agricultural activities is ligninocellulose, which is composed of cellulose, hemicellulose and lignin (Sánchez, 2009). Lignin forms a barrier to protect cellulose and hemicellulose from enzymatic attack. Under natural conditions, WRF can attack the lignin barrier to obtain energy from cellulose, via secretion of hydrolytic ligninolytic enzymes, including lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases. In this study, *G. lucidum* laccase production and activity was determined using submerged culture fermentation.

It has been reported that the carbon source is the most important factor in laccase production, and that the addition of suitable amounts of other sugars to the culture media has a benign influence on laccase synthesis (Teerapatsakul et al., 2007). In this study, we observed that low concentrations of glucose were the optimal carbon source for *G. lucidum* laccase production. The other tested sugars and high concentrations of sugars reduced laccase production when they were compared with low concentration of glucose. At high concentrations, sugars may satisfy the nutrient demands of *G. lucidum* for biomass growth, without the requirement to secret laccase to degrade lignin in order to obtain energy from cellulose. *Trametes pubescens* laccase synthesis is also repressed when glucose exceeds a certain concentration (Galhaup et al., 2002). Additionally, it has been reported that high concentrations of glucose can trigger the synthesis of extracellular polysaccharides which can interfere with the extraction of laccase from the culture broth (Eggert et al., 1996).

The optimal nitrogen source conditions for laccase production observed in this work were different to previous reports in other fungi. In some WRF species, such as *Pycnoporus cinnabarinus* and *Botryosphaeria sp.*, nitrogen limitation is required for the optimal production of laccase (Eggert et al., 1996; Vasconcelos et al., 2000), and in contrast, nitrogen-rich culture conditions were necessary for high levels of laccase in *Physisporinus rivulosus* (Hildén et al., 2007) and *Trametes pubescens* fermentation (Galhaup et al., 2002). In this study, laccase synthesis by *G. lucidum* was enhanced in nitrogen-rich culture conditions using yeast extract as a sole nitrogen source. Yeast extract is known to enhance laccase synthesis and our results were in agreement with the optimal culture conditions for *Ganoderma sp*. (Teerapatsakul et al., 2007). Yeast extract consists primarily of amino acids, peptides,

Table 1. Summary of laccase purification from *Ganoderma lucidum.*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude culture broth	5812.35	1063.40	5.46	1.0	100
$(NH_4)_2SO_4$ precipitation	5254.35	479.40	10.96	2.0	90.39
DEAE FF 16/10	2188.73	103.58	21.13	3.87	37.66
Sephacryl S-100 HR	1781.40	45.38	39.25	7.19	30.65

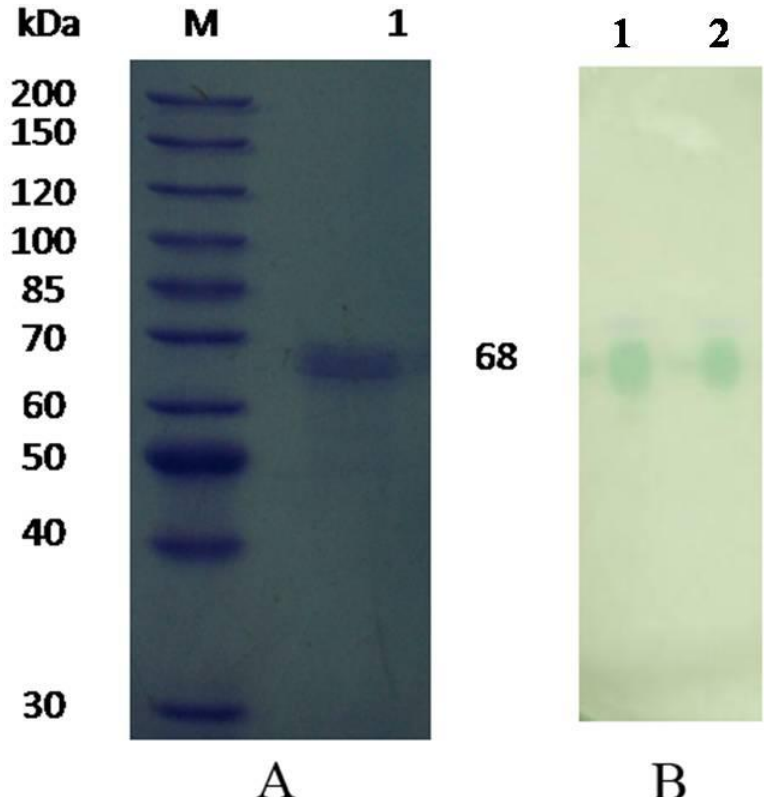

Figure 4. SDS-PAGE and zymogram analysis of laccase purified from *Ganoderma lucidum*. (A) SDS-PAGE gel stained with Coomassie blue R-250; M, low molecular mass protein marker; lane 1, purified laccase. (B) Native PAGE gel stained with 1 mM ABTS; lane 1, purified laccase; lane 2, laccase crude extract.

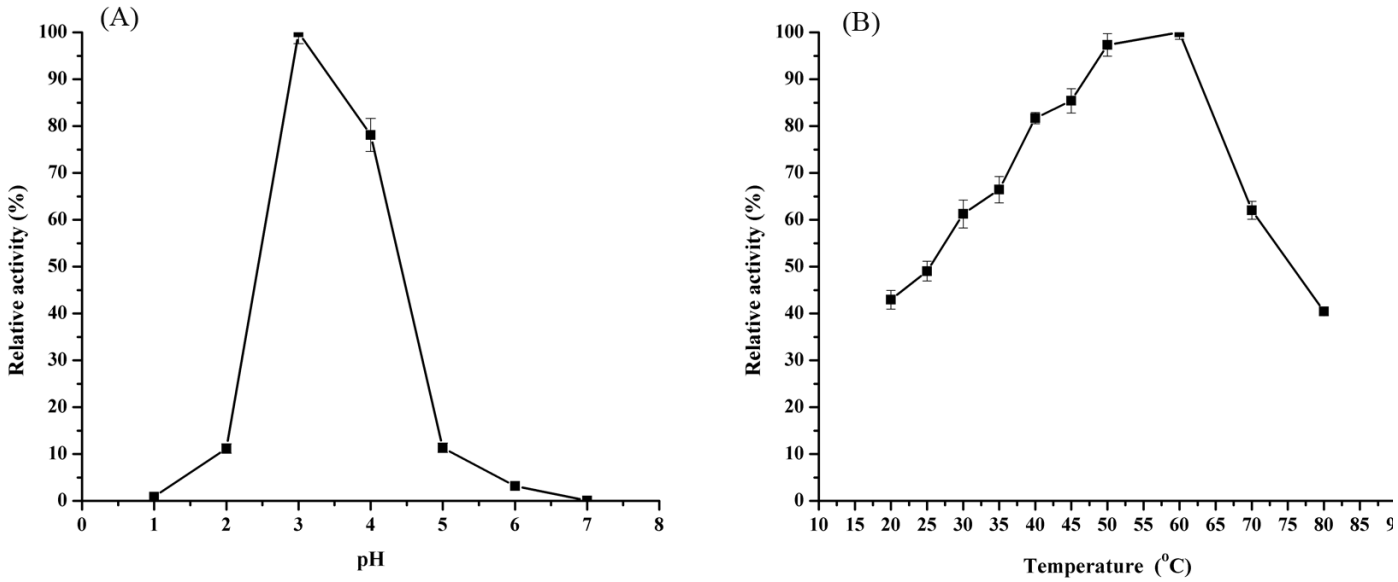

Figure 5. Effect of pH and temperature on the activity of laccase purified from *Ganoderma lucidum*. (A) The effect of pH on laccase activity at 30°C using ATBS as a substrate. (B) The effect of temperature on laccase activity at pH 3.0 using ATBS as a substrate.

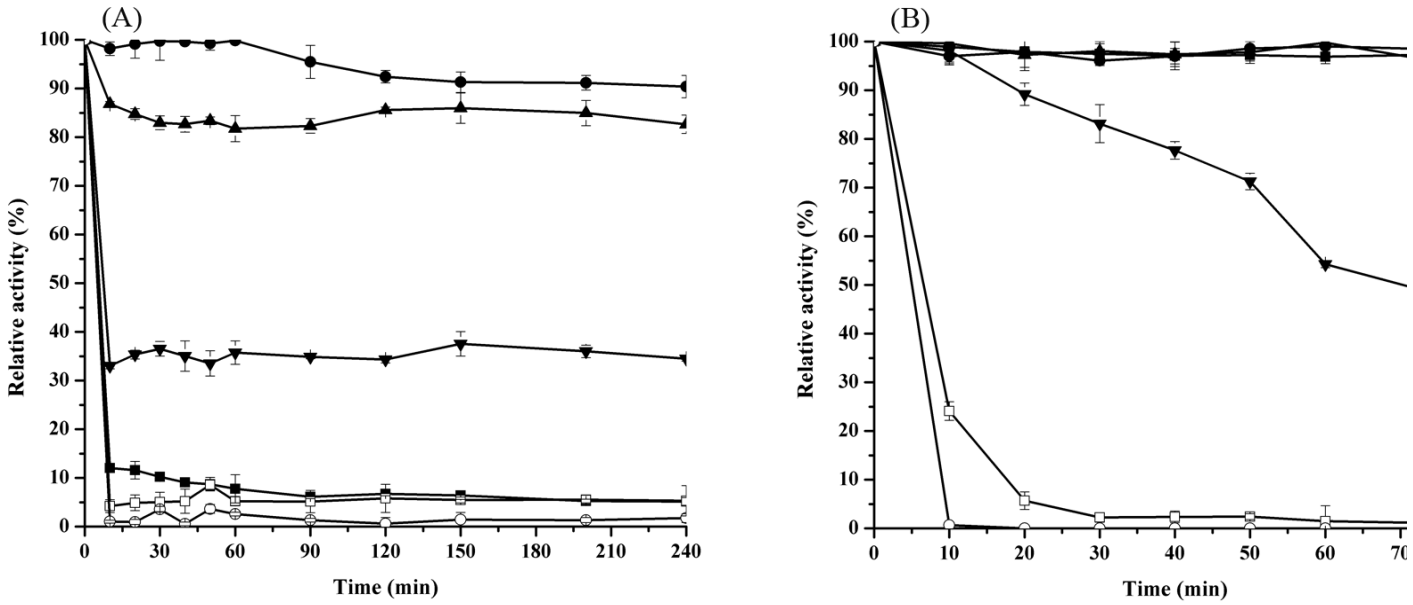

Figure 6. The pH and thermal stability of laccase purified from *Ganoderma lucidum*. (A) pH stability over 240 min using ATBS as a substrate: ■, pH 2.0; ●, pH 3.0; ▲, pH 4.0; ▼, pH 5.0; □, pH 6.0; ○, pH 7.0. (B) Thermal stability over 80 min using ATBS as a substrate: ■, 30°C; ●, 40°C; ▲, 50°C; ▼, 60°C; □, 70°C; ○, 80°C.

nucleotides and other soluble components of yeast cells (Chae et al., 2001), which may explain why yeast extract can stimulate laccase production more effectively than other organic nitrogen sources.

It has been reported that the abundant nutrients in agro-industrial wastes can be used to maintain the growth of microorganisms in fungal enzyme fermentation processes (Hatvani and Mécs, 2001). Murugesan et al. (2007) reported that wheat bran stimulated a high level of laccase production in *G. lucidum* fermentation, which was attributed to the presence of phenolic acids (Hegde et al., 2006; Murugesan et al., 2009b). Ferulic acid, syringic

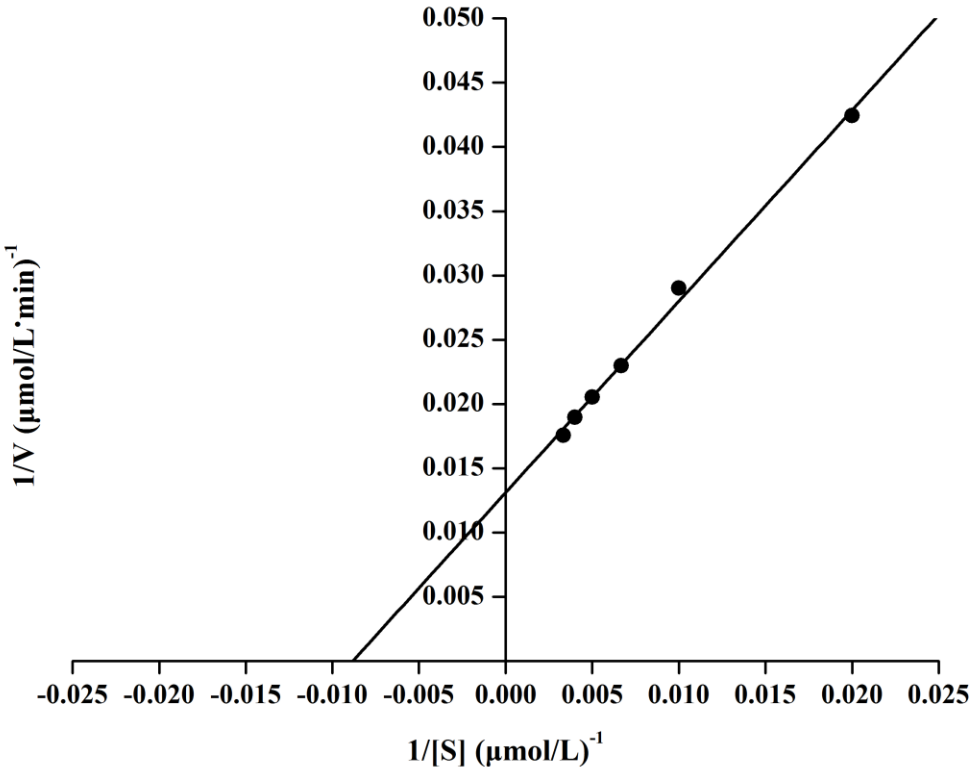

Figure 7. The Lineweaver-Burk plot of laccase purified from *Ganoderma lucidum* in different concentration of ABTS.

acid and coumaric acid are natural phenolic acids which stimulate laccase production in WRF (Farnet et al., 2004; Revankar and Lele, 2006).

Apart from wheat bran, other agricultural wastes can stimulate laccase biosynthesis, and many studies have indicated that fruit peels can be added to culture media as lignocellulosic substrates to enhance the production of laccase (Lorenzo et al., 2002; Elisashvili et al., 2006; Rosales et al., 2007). Like wheat bran, fruit peels contain high levels of sugars, cellulose, protein and lignin (Grohmann et al., 1995), which can maintain the growth of *G. lucidum*. In this study, pomelo peel was the optimal lignocellulosic substrate for laccase production in submerged fermentation, compared to other fruit peels and wheat bran. We observed that the peels of the *Rutaceae* family can significantly induce laccase production by *G. lucidum*. Sathishkumar et al. (2010) reported that banana peel resulted in optimal laccase production; however, these observations are in disagreement with our results, as banana peel inhibited *G. lucidum* laccase production.

Many fungal laccase enzymes have been purified and their molecular properties studied (Table 2). Fungal laccases have a number of different isoforms with a molecular mass ranging from 40 to 80 kDa (Eggert et al., 1996; Wang et al., 2010), and this variation could be attributed to the different ecological origins of each species or culture conditions. It has been reported that *G. lucidum* produces two isoforms of 40 and 68 kDa (D'Souza et al., 1999); however, the molecular weight of laccase was determined to be 43 kDa by other investigators (Murugesan et al., 2007). In this study, native-PAGE and SDS-PAGE analysis confirmed that we purified single laccase isoform of 68 kDa from *G. lucidum* fermentation.

Laccase can oxidize a wide range of substrates including phenolic and non-phenolic substrates; however, most reports suggest that fungal laccase prefers ABTS as the ideal substrate (Hildén et al., 2007; Wang et al., 2010). Therefore, we used ABTS to obtain the kinetic constant values, and the K_m value obtained for *G. lucidum* laccase is similar to the K_m values of the laccases from *Lentinula edodes* (Nagai et al., 2002) and *Pycnoporus sanguineus* (Vite-Vallejo et al., 2009).

Many reports have shown that the optimal pH for laccase varies when different substrates are used (Table 2); however, using ABTS as a substrate many laccase enzymes exhibit an optimal catalytic pH value in the acidic range (Halaburgi et al., 2011; Wang and Ng, 2006b), which is consistent with this study as the optimal pH was 3.0. Raising the pH value above 4.0, or below 3.0, rapidly decreased laccase activity, indicating that *G. lucidum* laccase is relatively stable at pH 3.0 to 4.0 and has a narrow range of pH stability.

Table 2. Comparison of the partial properties of different laccase enzymes isolated from white rot fungi.

Source of laccase	Optimal pH	Optimal temperature (°C)	$t_{1/2}$(h)	K_m(mM)	K_{cat}(S-1)	K_{cat}/K_m(mM^{-1} S^{-1})	Reference
Physisporinus rivulosus Lac-3.5	3.0 (DMP) 3.0 (Syringaldazin) 3.5 (Guaiacol) 2.0 (ABTS)	n.d.	70°C = 1	DMP = 0.088 Syringaldazin = 0.027 Guaiacol = 2.095 ABTS = 0.011	DMP=347 Syringaldazin = 282 Guaiacol=71 ABTS=286	DMP =390 Syringaldazin=1500 Guaiacol=64 ABTS=2700	(Hildén et al., 2007)
Physisporinus rivulosus Lac-4.8	3.0 (DMP) 3.0 (Syringaldazin) 3.5 (Guaiacol) 2.0 (ABTS)	n.d.	70°C = 0.5	DMP = 0.084 Syringaldazin = 0.003 Guaiacol = 1.406 ABTS = 0.017	DMP=481 Syringaldazin=124 Guaiacol=82 ABTS=409	DMP=570 Syringaldazin=4400 Guaiacol=5.8 ABTS=2400	(Hildén et al., 2007)
Pycnoporus sp. SYBC-L1 Lac I	3.5 (DMP) 5.0 (Syringaldazin) 4.0 (Guaiacol) 3.0 (ABTS)	70 (DMP) 45 (syringaldazin) 70 (guaiacol) 65 (ABTS)	50°C= 69.31 60°C = 2.58 70°C = 0.13 80°C = 0.07	DMP = 0.3410 Syringaldazin = 0.1670 Guaiacol = 1.1250 ABTS = 0.0166	DMP=188.07 Syringaldazin=123.89 Guaiacol=98.15 ABTS=326.03	DMP=551.52 Syringaldazin=741.86 Guaiacol=87.24 ABTS=19640.36	(Wang et al., 2010)
Pycnoporus sp. SYBC-L1 Lac II	3.0 (DMP) 5.5 (Syringaldazin) 3.5 (Guaiacol) 2.5 (ABTS)	75 (DMP) 50 (syringaldazin) 50 (guaiacol) 70 (ABTS)	50°C = 256.72 60°C = 21.00 70°C = 2.06 80°C = 0.17	DMP = 0.1928 Syringaldazin = 0.0891 Guaiacol = 0.7452 ABTS = 0.0435	DMP=737.48 Syringaldazin=357.14 Guaiacol=176.87 MABTS=1356.01	DMP=3825.10 Syringaldazin=4008.31 Guaiacol=237.35 ABTS=31172.64	(Wang et al., 2010)
UD (unidentified fungal isolate)	6.0 (DMAB MBTH) 4.5 (Syringic acid) 6 (Guaiacol) 4 (ABTS)	n.d.	n.d.	DMAB MBTH = 6.90 Syringic acid = 0.026 Guaiacol = 0.251 ABTS = 0.0123	DMAB MBTH=368 Syringic acid =10.1 Guaiacol=42.1 ABTS=778	DMAB MBTH=53 Syringic acid =388 Guaiacol=168 ABTS=63300	(Jordaan et al., 2004)
Clitocybe maxima	3.0 (ABTS)	60 (ABTS)	n.d.	ABTS = 0.0617	n.d.	n.d.	(Zhang et al., 2010)
Pleurotus ostreatus strain 10969	4.0 (ABTS)	50 (ABTS)	n.d.	ABTS = 0.31	n.d.	n.d.	(Liu et al., 2009)
Agaricus blazei	5.5 (DMP) 5.5 (Syringaldazin) 2.3 (ABTS)	n.d.	n.d.	ABTS=0.063 DMP=1.026 Syringaldazin=0.004 Guaiacol=4.307	ABTS=21 DMP=15 Syringaldazin=5 Guaiacol=159	ABTS=340 DMP=14 Syringaldazin=1228 Guaiacol=4	(Ullrich et al., 2005)
Lentinula edodes	4.0 (ABTS) 5.0 (p-phenylenediamine) 4.0 (Pyrogallol) 4.0 (DMP) 4.0 (Guaiacol) 5.0 (Ferulic acid) 4.0 (Catechol)	40 (ABTS)	n.d.	ABTS=0.108 p-phenylenediamine=0.256 Pyrogallol=0.417 DMP=0.557 Guaiacol=0.917 Ferulic acid=2.86 Catechol=22.4	n.d.	n.d.	(Nagai et al., 2002)

Table 2. Contd.

Pycnoporus sanguineus CelBMD001	4.0 (ABTS)	80 (ABTS)	30°C = 20 40°C = 14 50°C = 5 60°C = 2	ABTS=0.106 Syringaldazin=0.102 Guaiacol=0.25 *o*-dianisidine=0.044	ABTS=59.28 Syringaldazin=39.86 Guaiacol=2.05 *o*-dianisidine=9.4	ABTS=550 Syringaldazin=383.33 Guaiacol=8.17 *o*-dianisidine=218.33	(Vite-Vallejo et al., 2009)
Ganoderma lucidum	3.0 (ABTS)	60 (ABTS)	n.d.	ABTS=0.114	ABTS=74.63	ABTS=654.65	This work

n.d., Values not determined.

Many thermostable laccase enzymes have been reported in other fungi (Table 2), including *Pycnoporus* sp. (Wang et al., 2010), *Cladosporium cladosporioides* (Halaburgi et al., 2011), *Pleurotus eryngii* (Wang and Ng, 2006a), *Physisporinus rivulosus* (Hildén et al., 2007), *Tricholoma giganteum* (Wang and Ng, 2004) and *Pleurotus ostreatus* (Liu et al., 2009). *G. lucidum* is an important member of the WRF and laccase from this species of fungi are currently used to decolorize some reactive dyes (Murugesan et al., 2009a); however, the production of thermostable laccase by *G. lucidum* has not received intensive investigation. Murugesan et al. (2007) suggested that submerged fermentation using wheat bran lead to production of thermostable laccase by *G. lucidum*; however, in this study, we produced a highly thermo-tolerant laccase in submerged fermentation using pomelo peel instead of wheat bran.

In conclusion, this study demonstrates that *G. lucidum*, an important white-rot fungus, produced laccase as the main ligninolytic enzyme. The laccase production of *G. lucidum* was stimulated by lignocellulosic substrates, especially the peels of the *Rutaceae* family. The purified laccase was characterized and the results showed that laccase produced by *G. lucidum* was thermostable. These characteristics suggested that *G. lucidum* possessed great potential applications for both industry and biotechnology.

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