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Laccase-catalyzed oxidation of phenolic compounds in organic media

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ABSTRACT

Rhus vernificera laccase-catalyzed oxidation of phenolic compounds, i.e., (+)-catechin, (−)-epicatechin and catechol, was carried out in selected organic solvents to search for the favorable reaction medium. The investigation on reaction parameters showed that optimal laccase activity was obtained in hexane at 30 °C, pH 7.75 for the oxidation of (+)-catechin as well as for (−)-epicatechin, and in toluene at 35 °C, pH 7.25 for the oxidation of catechol. E_a and Q_{10} values of the biocatalysis in the reaction media of the larger log *p* solvents like isooctane and hexane were relatively higher than those in the reaction media of lower log *p* solvents like toluene and dichloromethane. Maximum laccase activity in the organic media was found with 6.5% of buffer as co-solvent. A wider range of 0–28 μg protein/ml in hexane than that of 0–16.7 μg protein/ml in aqueous medium was observed for the linear increasing conversion of (+)-catechin. The kinetic studies revealed that in the presence of isooctane, hexane, toluene and dichloromethane, the K_m values were 0.77, 0.97, 0.53 and 2.9 mmol/L for the substrate of (+)-catechin; 0.43, 0.34, 0.14 and 3.4 mmol/L for (−)-epicatechin; 2.9, 1.8, 0.61 and 1.1 mmol/L for catechol, respectively, while the corresponding V_{max} values were 2.1×10^{-2}, 2.3×10^{-2}, 0.65×10^{-2} and 0.71×10^{-2} δA/μg protein min); 1.8×10^{-2}, 0.88×10^{-2}, 0.19×10^{-2} and 1.0×10^{-2} δA/μg protein min); 0.48×10^{-2}, 0.59×10^{-2}, 0.67×10^{-2} and 0.54×10^{-2} δA/μg protein min), respectively. FT-IR indicated the formation of probable dimer from (+)-catechin in organic solvent. These results suggest that this laccase has higher catalytic oxidation capacity of phenolic compounds in suitable organic media and favorite oligomers could be obtained.

1. Introduction

Plant phenols have drawn attention due to their multiple biological effects including antioxidant, anti-mutagenic, anti-carcinogenic, anti-viral and anti-inflammatory activity [1–3]. These activities enable natural phenolic compounds to reduce carcinogenesis [4] and hardening arteries [5]. However, phenols occurring in fruit and vegetable are responsible for browning and consequently, the decline of sensor, nutritional and flavor quality of either the raw product or the processed food [6,7]. The removal of phenolic compounds could improve the sensory quality of the products and generally, it is done through the polymerization with oxidative enzymes without hazardous effect [8,9]. In addition, many reports have shown that high molecular weight polyphenols exhibit enhanced biological properties over the last decade [10]. Examples as the polymer of rutin showed greatly improved superoxide scavenging activity and inhibition effects on human low-density lipoprotein (LDL) compared with the rutin monomer [11]; polycatechin also presented a much higher superoxide scavenging activity than the monomeric catechin did [12], indicating enzyme-catalyzed oxidative polymerization of phenolic compounds to be an important approach to produce new substance of therapeutic significance.

Laccases (EC 1.10.3.2) are among effective enzymes capable to catalyze the oxidation of phenols, which produce water as the only one by-product making it an ideally 'green' enzyme. Laccase shows less substrate specificity than tyrosinase based on the fact that *o*-, *p*-diphenols undergo biotransformation by laccase strongly [13], whereas tyrosinase uses *o*-diphenol as exclusive substrate [14]. Hence laccase has become a focus for enzymatic polymerization of phenols.

Reaction media play an important role on enzymatic catalysis. Generally, biocatalysis is conducted in non-organic or aqueous–organic biphasic systems. The latter case is used to

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improve solubility of hydrophobic substrate, and to enhance enzymatic stability as well as to manipulate the reaction constant [15,16]. Tse et al. showed that the organic media facilitated the polymerization of phenolic compounds by tyrosinase. The specific activity (SA) of the enzyme for catechin bioconversion in dichloromethane ($\log p = 2.0$) medium is as 20 orders of magnitude as that in aqueous solution [17]. Further studies reported that this SA in heptane ($\log p = 4.0$) is even 3 times higher than in dichloromethane [18].

Superactivity of laccase in water-restricted media has also been reported in various reverse micelles for the biotransformation of phenolic environmental pollutants and lignin-related compounds [13,19,20]; however, few studies have been done on plant phenolic compounds. The purpose of this present work is to investigate the ability of laccase-catalyzed biotransformation of natural phenolic compounds in water-restricted media, and specifically, to determine the optimum reaction parameters and the kinetic characteristics of laccase, using selected phenolic substrate as models.

2. Materials and methods

2.1. Materials

Laccase from *Rhus vernificera*, 120 U (85 ± 1.5 μg protein)/mg solid, was purchased from Sigma Chemical Co. (St-Louis, MO). One unit is defined as a δA530 of 0.001 per min at pH 6.5 at 30 °C in a 3-ml reaction volume using syringaldazine as substrate. Chloroform, dichloromethane, toluene, hexane, isooctane were from Fisher Scientific Co. (Pittsburgh, PA). (+)-Catechin, (−)-epicatechin, catechol and chlorogenic acid were from Sigma Chemical Co. All organic solvents used were dried overnight with anhydrous sodium sulfate prior to use.

2.2. Preparation of stock solution

Stock substrates were prepared of (+)-catechin (200 mmol/L) and (−)-epicatechin (200 mmol/L) in methanol, catechol (400 mmol/L) in ethanol, chlorogenic acid (100 mmol/L) in methanol. Each stock solution was prepared daily just before use and homogenized using a Branson water-bath sonicator (Branson, Danbury, CT). A 0.25-mg/L stock enzyme was made by suspending enzyme protein in citric phosphate buffer with pH 6.5 (0.1 mol/L) according to the manufacturer's protocol for the experiments of the effect of solvents, temperature and water content (2.3–2.5), or with selected optimum pH value for the remaining trials (2.6–2.9).

2.3. Selection of solvents

Laccase-catalyzed reactions were carried out in chloroform ($\log p = 2.0$), dichloromethane ($\log p = 2.0$), toluene ($\log p = 2.5$), hexane ($\log p = 3.5$) and isooctane ($\log p = 4.5$) to screen favorable organic solvents for the oxidation of phenolic compounds. In order to make a reaction mixture of initial substrate concentrations of 5, 5, 10, 2.5 mmol/L for (+)-catechin, (−)-epicatechin, catechol and chlorogenic acid, respectively and initial enzyme concentration of 12.5 μg/ml, 740 μl of solvent were mixed with 20 μl of substrate stock solution under stirring in vortex (Model G-560, Scientific Industries, Bohemia, NY, USA) for 2 s, and immediately 40 μl of laccase-buffer suspension were added. Parallel contrast reactions with buffer solution instead of enzyme suspension for each of the solvent were performed; and so did reference reactions with buffer alone instead of organic solvent for each of the substrate. The reaction mixture was kept under agitation for 1 h at 25 ± 1 °C. At the end of reaction, each mixture was transferred to a 1.5-ml centrifugation tube and dried in Speedvac (Savant Instrument, Inc., Holbrook, NY, USA) till yellow solid samples were obtained. The samples were dissolved in 800 μl methanol. The UV–vis profiles of the yellow pigments resulting from the laccase-catalyzed end products in the reaction media was measured spectrophotometrically from 300 to 800 nm using a Beckman DU650 UV–vis spectrophotometer (Beckman Coulter, Inc., USA). Each scanning profile was plotted with the average values of duplications.

2.4. Effect of temperature on laccase activity

The effect of temperature on laccase activity was assayed at temperature ranging from 20 to 60 °C with the same performance described previously in different solvents. (+)-Catechin was used as substrate. The reaction mixture was dried and resuspended in methanol for testing absorbance at 433 nm. Each reaction was repeated three times, and parallel blank assay with buffer solution instead of enzyme suspension was also determined.

2.5. Effect of water ratio on laccase activity

The effect of water on the laccase biocatalysis reaction of (+)-catechin was investigated by using different amounts of water as co-solvent in the enzymatic reaction mixture. The concentration of water used in the reaction media ranged from 1.0 to 100% (v/v) for hexane.

2.6. Effect of pH on laccase activity

Same reactions as that for the trials of the effect of temperature (2.4) were carried out at the selected temperature and water ratio of 6.5% to determine the effect of pH on laccase activity by preparing both the stock (+) substrate and enzyme suspension at a range of pH 4.0–8.0 in citric phosphate buffer (0.1 mol/L). Read absorbance at 433, 440, 388 nm for the end product from (+)-catechin, (−)-epicatechin and catechol, respectively.

2.7. Effect of enzyme protein concentration on laccase catalysis

Enzyme protein ranging from 0.2 to 36 μg/ml was employed in different reactions to determine the effect of protein dosage on laccase catalysis, using (+)-catechin as substrate both in the reaction media of hexane and buffer.

2.8. Determination of kinetic parameters

K_m and V_{max} for the laccase biocatalysis reaction in organic solvent media were determined by using different concentrations of substrate from 0.25 to 2.0 mmol/L, from 0.2 to 3.0 mmol/L and from 0.25 to 7.5 mmol/L for (+)-catechin, (−)-epicatechin and catechol, respectively. End products were dried and redissolved in methanol to test absorbance at 433 nm for (+)-catechin, at 440 nm for (−)-epicatechin and at 415 nm for catechol in every 5, 10, 20, 30, 45 and 60 min, respectively.

2.9. End product preparation and its identification with FT-IR

Repeat the laccase-catalyzed oxidation of (+)-catechin, (−)-epicatechin and catechol under the selected optimum condition with magnified substrate amount of 30 mg or so, 3 repeats were set to benefit yield calculation. The colored end product resulting in each organic solvent were isolated from the freeze dried reaction mixture by size exclusion chromatography (SEC), using Trisacryle GF 05 M (Biosepra Inc., Marlborough, MA) column and

eluting with 50% acetone. Condense and dry the product with Speedvac, freeze-dryer (Labconco, USA) in turn. Weigh each dried sample to calculate the yield of end product. The infrared spectroscopic analyses were performed with a Varian model 600 UMA Fourier transform infrared spectroscopy (FT-IR) spectrometer (Varian Inc., USA) equipped with a deuterated triglycine sulfate (DTGS) detector. The samples of catechin as well as each of its pre-milled products were deposited separately on the surface of a horizontal attenuated total reflectance (ATR) sampling accessory. FT-IR spectra were recorded by 128 scans at 4 cm^{-1} resolution.

2.10. The calculation of the results

Enzyme activities in each reaction were expressed as SA which was defined as the change in absorbance unit (δA) at the maximum absorption wavelength (λ_{max}) of the end product per μg enzyme protein per min in a 1-ml reaction volume.

Activation energy (E_a) was calculated according to the equation [23]: slope of Arrhenius plot = E_a/RT, where R is the gas constant (8.3145 kJ mol^{-1} K^{-1}); T is the absolute temperature (K).

3. Results and discussion

3.1. Effect of organic solvents on laccase catalysis

Fig. 1 shows the UV–vis profiles of the colored products originating from the enzymatic oxidation end products of (+)-catechin, (−)-epicatechin and catechol in the selected organic reaction media. The results present distinct maximum absorption peaks at 433 and 440 nm for the enzymatically produced colored substances from (+)-catechin and (−)-epicatechin, respectively, in the reaction media of hexane, isooctane, toluene and dichloromethane

$$SA = \frac{\text{O.D.} \times \text{volume of total reaction mixture (ml)}/1\,\text{(ml)}}{\text{volume of protein suspension added (ml)} \times \text{protein content of laccase suspension (μg/ml)} \times \text{reaction time (min)}} (\delta A/\mu g\,\text{protein} \cdot \text{min}))$$

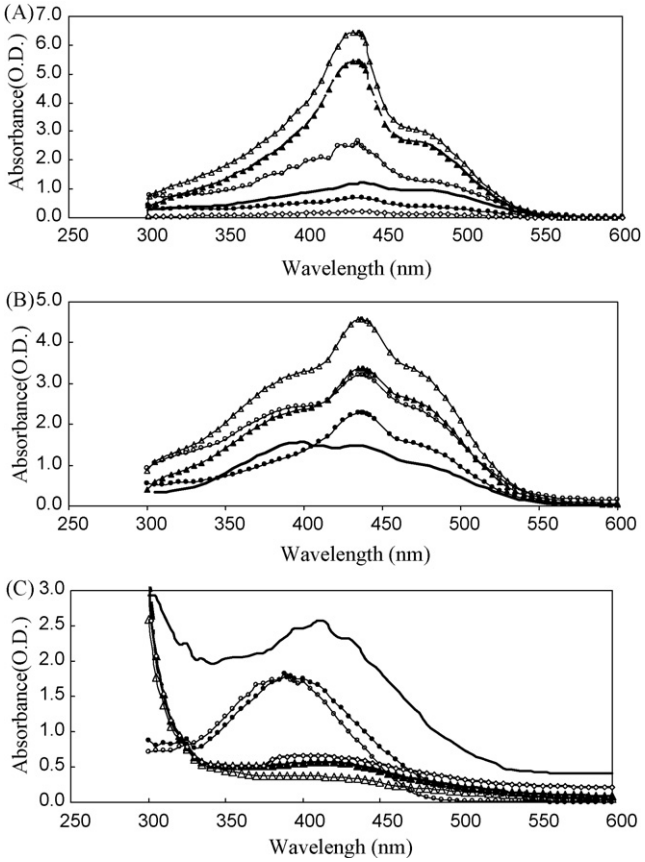
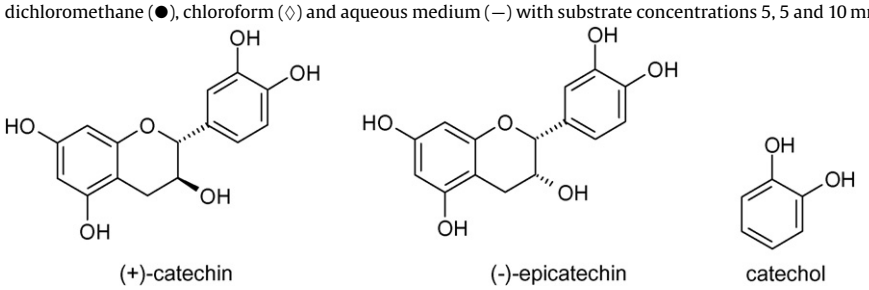


Fig. 1. Scanning profiles of the end products from laccase-catalyzed oxidation of (+)-catechin (A), (−)-epicatechin (B), and catechol (C) in hexane (△), isooctane (▲), toluene (○), dichloromethane (●), chloroform (◊) and aqueous medium (—) with substrate concentrations 5, 5 and 10 mmol/L for (+)-catechin, (−)-epicatechin and catechol, respectively.



except chloroform. In contrast, the results show that in the absence of the enzyme in the reaction mixture, there was no indication of these peaks.

Moreover, laccase biocatalysis of (+)-catechin in organic media produced the end products with the same maximum absorption as that obtained in aqueous medium (Fig. 1A); in case of laccase oxidation of epicatechin, they are quite different. End-products with different maximum absorption wavelengths from (+)-catechin obtained by *Myceliophthora* laccase biocatalysis were previously reported by Kurisawa et al. who explained the difference in molecular weights among products from the same substrate attribute to the difference of their visible spectra [12]. The findings suggest that the resulting end products in organic solvents are different from that in aqueous medium with epicatechin rather than catechin.

Fig. 1A and B also shows that the enzymatic conversion are higher in most organic solvents than that in aqueous medium, and decrease with the increase of solvent polarity index as hexane (0), isooctane (0.1), toluene (2.4) and dichloromethane (3.1). In case of chloroform, its hydrophobicity values ($\log p = 2.0$) is the same as dichloromethane, whereas its polarity index (4.4) is more than that of dichloromethane (3.1), almost resulting in no end product for substrates of (+)-catechin and (−)-epicatechin. These results indicate clearly that those solvents with polarity index higher than 3.1 are not suitable for laccase catalysis in water-restricted organic solvent media, when using catechin or epicatechin as substrate. In addition, solvents with a $\log p$ value lower than 2.0 were reported not to be suitable for phenol polyphenol oxidase (PPO) catalysis [18], as they may strongly distort the essential water layer required to maintain the enzyme in its native catalytically active conformation. The findings in this research suggest, however, as for laccase it is more practical using polarity index rather than $\log p$ to indicate the suitability of solvents as solvents with same $\log p$, but different polarity exhibited obviously different effect on enzyme activity.

With respect to the substrate catechol, the influence of selected organic solvents almost changed to opposite with the results obtained with substrates of catechin and epicatechin. The absorbance for end products derived from catechol decreased all to lower than that in aqueous medium, showing more declination in larger $\log p$ solvents like hexane and isooctane than in lower $\log p$ solvents like dichloromethane and toluene. Similar conclusion about the role of organic solvents with catechol oxidation was also obtained by other researchers with tyrosinase as catalyst, where the behaviors in the different solvents were attributed to the effect of polarity on the conformation of the enzyme as well as the solubility of substrate and/or products [14]. The maximum absorption of end products from catechol in hexane and isooctane appeared at the same wavelength as that in aqueous medium (415 nm); however, it changed to 388 and 390 nm in toluene and dichloromethane, respectively.

No reaction occurred for chlorogenic acid in the reaction media of dichloromethane, toluene, hexane and isooctane because the substrate pre-dissolved in methanol precipitated when mixed with any of the four organic solvents. It can be concluded that the substrate will react with enzyme in organic–aqueous medium only when it is soluble.

3.2. Effect of temperature on laccase catalysis

The results show that the profile of laccase activity with temperature, using (+)-catechin and catechol as substrates, is all a bell-shape curve, whose maximum values indicate the optimal temperature of 30 °C in the reaction media of hexane as well as isooctane, 35 °C in toluene as well as dichloromethane for *R. vernificera* laccase catalysis.

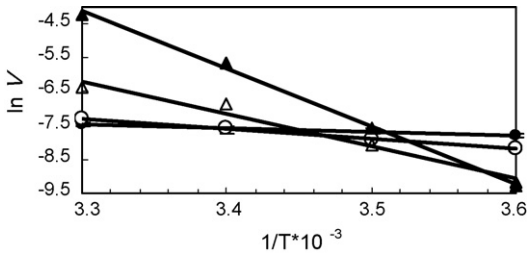


Fig. 2. Arrhenius plot of laccase-catalyzed catechin oxidation in the reaction media of hexane (△), isooctane (▲), toluene (○) and dichloromethane (●).

Activation energy (E_a) of the laccase-catalyzed oxidation of (+)-catechin calculated from the Arrhenius plot (Fig. 2) was 27.0, 44.0, 23.9 and 19.1 kJ/mol in the reaction media of hexane, isooctane, toluene and dichloromethane, respectively, and their coefficient (Q_{10}) at the optimum temperature was 1.11, 1.20, 1.09 and 1.04, respectively. These findings suggest that the laccase-biocatalysis rate in the reaction media of the larger $\log p$ solvents like isooctane and hexane are relatively high in a temperature dependent manner, as indicated by their relatively high E_a and Q_{10} values. Moreover, the intrinsic characteristics of the organic solvents were reported to influence the optimal temperature of enzyme catalysis [18]. In this study solvents with higher viscosity show more temperature-dependant trend such as isooctane (0.53) to hexane (0.33), toluene (0.59) to dichloromethane (0.44), thereby amending the analysis that the $\log p$, viscosity, and boiling point of the organic solvents affected enzymatic catalysis temperature [18].

3.3. Effect of water ratio on laccase activity

Water mixed with organic solvent is essential for the solubility of enzyme in the reaction media, therefore we examined the effect of water as co-solvent on the laccase–catechin oxidative reaction in selected organic solvent media (Fig. 3). The laccase–(+)-catechin

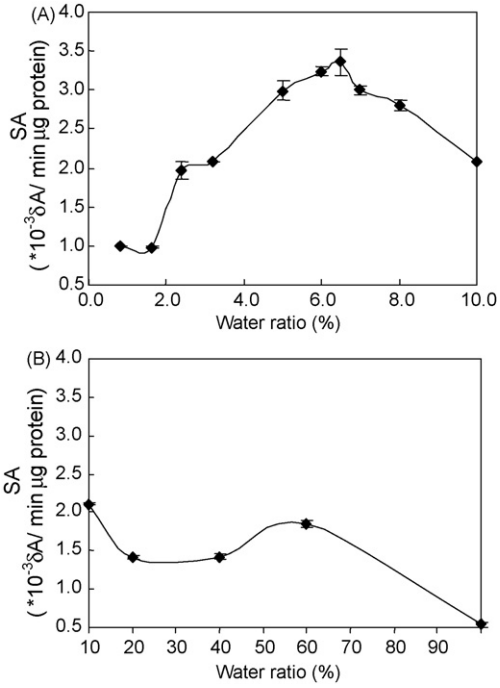


Fig. 3. Effect of the water ratio in organic media system on laccase activity. (A) Water ratio is less than 10%; (B) water content is between 10 and 100%.

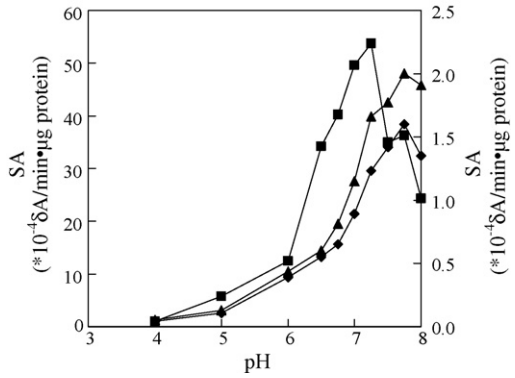


Fig. 4. Effect of pH on laccase-catalyzed oxidation of (+)-catechin in hexane (▲), (−)-epicatechin in hexane (♦) and catechol in toluene (■).

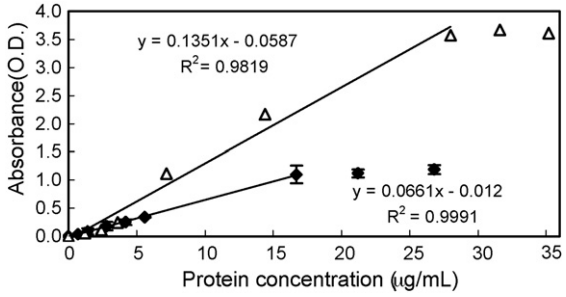


Fig. 5. The effect of the protein concentration on laccase biocatalysis in the reaction media of buffer (♦) at 45 °C, pH 7.75 and hexane (△) at 25 °C, pH 7.75.

oxidative reaction in the hexane reaction medium showed higher activity with water content in biphasic mixture ranging from 5.0 to 7.0% (v/v), with a maximum point at 6.5% (Fig. 3A). The trend of enzyme activity was decreased in the reaction medium when water ratios were changed in opposite (Fig. 3B). In reverse micelles (W/O), the enzyme activity displayed a bell-shaped dependence of hydration ratio. Ruckenstein and Karpe reported that superactivity existed for low substrate portioning in the surfactant layer; subactivity for high partitioning [24]. We can understand that in water restricted aqueous–organic media, the partition of substrate between organic solvent and a water layer around enzyme is the main factor which decides the frequency of substrate to combine with the enzyme, thereby influencing the enzymatic activity. Superficially, substrate was condensed and gathered around the enzyme, which may consequently enhance the enzyme activity, whereas, it is not a simple effect of condensation because the reaction resulted in only about 1/2 of the absorbance value when the organic solvent (hexane) was removed from the medium. The favorite conformation of the enzyme might be kept better in water-restricted non-polar organic medium than that in aqueous medium, contributing the increase of enzyme activity.

3.4. Effect of pH on laccase activity

A dramatic increase of laccase activity SA from pH 4 to 7.75 for the oxidation of (+)-catechin and (−)-epicatechin was observed (Fig. 4), moreover, same trend from pH 4 to 7.25 presented with the substrate of catechol. Thereafter, each SA decreased, indicating the optimum pH value for laccase-catalyzed oxidation of (+)-catechin, (−)-epicatechin and catechol were pH 7.75, 7.75, 7.25, respectively, where laccase activity increased 3–4 folds in comparison to that at pH 6.5. This result was in close agreement with that from Hosny and Rosazza who reported a optimum pH 7.4 for hydroquinone-mediated oxidation of (+)-catechin with laccase from *R. vernificera* [21]. However, optimal pH 4.7 by Brenna et al. and pH 7.0 by Osman et al. were obtained for catechin oxidation with laccase from *Trametes versicolor* [9], *Aspergillus oryzae* [22], respectively. These results generally reveal that the optimum pH value of laccase activity for a same substrate oxidation would be quite different with laccase from different sources. Laccase from *R. vernifiera* is alkali-philic.

3.5. Effect of protein concentration on laccase activity

The rate of the (+)-catechin (1 mmol/L) oxidation represented by the absorbance (proportional to oxidation of substrate) increased linearly with the laccase protein concentration increase from 0 to 16.7 μg/ml in aqueous medium and to 28 μg/ml in hexane medium (Fig. 5), thereafter the increase slowed down or almost stopped. This result indicates that the enzyme may disperse better in organic solvent and hence the declination in enzyme–substrate combination potency caused by enzyme-gathering happens at higher protein concentration than that in aqueous medium.

3.6. Kinetic parameters for laccase biocatalysis

K_m values represent the affinity of substrate with enzyme, the less the K_m, the more the affinity. Thus, it was learned from Table 1 that (+)-catechin or (−)-epicatechin, showed a higher affinity with laccase in the reaction media of isooctane, hexane and toluene than that obtained in the dichloromethane reaction medium, as indicated by the respective K_m values of 0.77, 0.97, and 0.53 mM for (+)-catechin, and 0.43, 0.34 and 0.14 mM for (−)-epicatechin in comparison to those of 2.9 and 3.4 mM. On the

Table 1
Kinetic parameters of laccase-catalyzed oxidation of three phenolic substrates in selected organic solvent media

Substrate	Reaction media	Yield of end product ($mg \cdot mg^{-1}$ substrate)	K_m (mM)	V_{max} ($\delta A/\mu g$ protein·min) $\times 10^{-2}$)	Catalytic efficiency[a] ($\times 10^{-2}$)
(+)-Catechin	Isooctane	$0.86 \pm 2.6 \times 10^{-2}$	0.77	2.1	2.8
	Hexane	$0.91 \pm 1.7 \times 10^{-2}$	0.97	2.3	2.4
	Toluene	$0.42 \pm 1.2 \times 10^{-2}$	0.53	0.65	1.2
	Dichloromethane	$0.01 \pm 1.2 \times 10^{-3}$	2.9	0.71	0.24
(−)-Epicatechin	Isooctane	$0.68 \pm 2.0 \times 10^{-2}$	0.43	1.8	4.1
	Hexane	$0.80 \pm 1.1 \times 10^{-2}$	0.34	0.88	2.6
	Toluene	$0.68 \pm 4.5 \times 10^{-3}$	0.14	0.19	1.4
	Dichloromethane	$0.37 \pm 5.2 \times 10^{-3}$	3.4	1.0	0.30
Catechol	Isooctane	$0.09 \pm 2.3 \times 10^{-2}$	2.9	0.48	0.17
	Hexane	$0.08 \pm 5.8 \times 10^{-2}$	1.8	0.59	0.33
	Toluene	$0.32 \pm 1.7 \times 10^{-2}$	0.61	0.67	0.70
	Dichloromethane	$0.34 \pm 2.5 \times 10^{-2}$	1.1	0.54	0.51

[a] The catalysis efficiency was defined as the ratio of V_{max} to K_m. K_m, Michaelis–Menten saturation constant; V_{max}, maximum reaction rate of oxidation.

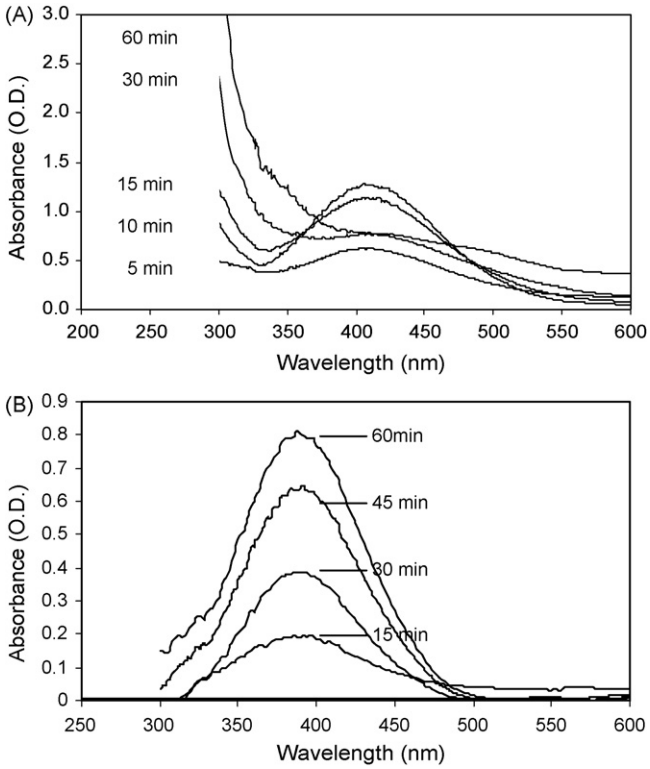


Fig. 6. Scanning profiles of end products from catechol at different time intervals in aqueous medium (A) at 45 ± 0.1 °C and dichloromethane (B) at 25 ± 1 °C. The initial substrate and enzyme concentrations in both reactions were 5 mmol/L and 14 μg/ml, respectively.

other hand, the catalytic efficiency, namely V_{max}/K_m ratio, was also used as an indication of the ability of enzyme to convert substrate into the corresponding end products under a given condition. Table 1 shows that the catalytic efficiency of laccase in the reaction media of isooctane, hexane and toluene was much higher than that obtained in the dichloromethane medium, using (+)-catechin or (−)-epicatechin as substrate. The overall findings show that maximal efficiency of laccase catalysis was demonstrated in the isooctane medium, followed by those of hexane and toluene; however, the lowest laccase–catechin and laccase-epicatechin biocatalysis were observed in the dichloromethane reaction medium. As for the substrate catechol, respective K_m value of 2.9, 1.8, 0.61, 1.1 mM and corresponding catalytic efficiency of 0.17×10^{-2}, 0.33×10^{-2} to 0.70×10^{-2}, 0.51×10^{-2} showed lower affinity with laccase and less catalytic efficiency in the presence of isooctane and hexane over toluene and dichloromethane, partially in agreement with the previous report on tyrosinase biocatalysis which revealed that tyrosinase showed a higher V_{max} and catalytic efficiency compared to that obtained with substrate catechol in the heptane reaction medium ($\log p = 4$) [12]. The enhancement of catechol-laccase catalysis in lower $\log p$ organic media over larger ones might be explained by the hydrophobicity of end product. The more hydrophobicity of the end product of catechol compared to that of (+)-catechins enables its partition in the organic phase with lower $\log p$, revealed by the detectable absorbance value of 0.2035, 0.1435 with the organic phase in toluene and dichloromethane reaction media, whereas the corresponding absorbance value for catechol in isooctane, hexane reaction medium as well as for substrate (+)-catechin in each organic medium were as less as 0.0013–0.0157. The inhibition effect of end product on laccase catalysis was thus reduced in reaction media of water-restricted toluene or dichloromethane as part of the end product was removed from aqueous phase to organic one, resulting in more catalytic efficiency.

Table 1 also shows that the yield of end product of each substrate from different solvent presented the trend of positive coefficient with the catalytic efficiency. (+)-Catechin in both hexane and isooctane as well as (−)-epicatechin in hexane resulted in the yield of 0.80 mg mg^{-1} above.

3.7. Effect of organic solvent on the end product of catechol

In the work we noticed that the color of reaction mixture of catechol in aqueous medium changed over reaction time. But the specific absorbance in visible spectrum (415 nm) did not show the expected subsequent increase. The scanning profile (Fig. 6A) displayed a consistent absorbance decay of the products in aqueous medium around 15 min after the reaction started and a consistent increase of the absorbance at wavelengths near to the violet spectrum. Specific absorbance peak in visible spectrum disappeared toward the end of the reaction. Catechol biotransformation by tyrosinase has been investigated well. It has been clear that

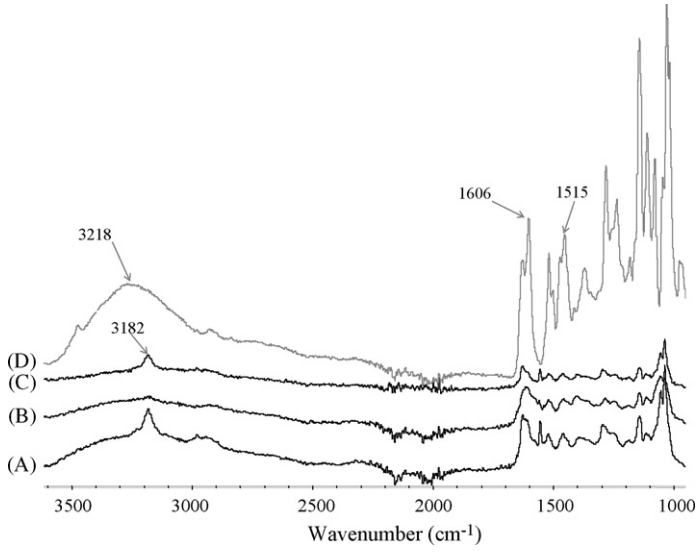


Fig. 7. FT-IR spectra of (+)-catechin (D) and its oxidation end products in the different reaction media of isooctane (A), hexane (B) and toluene (C).

its enzymatic oxidation undergoes two steps: first, an intermediate quinone is formed; second, the resulting quinone undergoes a series of non-enzymatic polymerization reactions leading to melanin [16]. The change in color and absorbance in this study confirmed that laccase-catalyzed oxidation of catechol occurred in the same way as tyrosinase in aqueous medium. Fortunately, when the reaction was performed in dichloromethane, the absorbance of yellow product increased till the end of the reaction for 60 min (Fig. 6B). It indicates that further polymerization of quinone does not occur in this organic solvent, making possible to obtain the intermediate product DOPA-quinone, which has therapeutic uses.

3.8. End product identification by FT-IR

The FT-IR spectrum of the substrate catechin (Fig. 7D) displays a weak and broad stretching band at 3218 cm^{-1} that corresponds to the hydroxyl groups involved in an intermolecular hydrogen bond and peak at 1606 cm^{-1} which corresponds to the C=C vibration of aromatic group. The absorption at 1515 cm^{-1} ascribed to the phenolic hydroxyl groups [17,25] is also observed. The absence of these characteristic absorptions in the spectra of each end product (Fig. 7A–C) except for a band still at 1606 cm^{-1} suggests that the structures of each end product were different from that of their parent substrates, though the aromatic ring existed. However, products resulting in the reaction media of isooctane and toluene may possess the same structure skeleton, suggested by two similar shaped FT-IR spectra, each of which presented a new sharp peak at 3182 cm^{-1}, attributed to the absorption of phenolic hydroxyl groups in the form of a dimer. These results indicate that catechin was mostly converted into its dimer in isooctane or toluene. Nevertheless, further identification is needed for the typical absorption at 3182 cm^{-1}, which disappear clearly in the spectrum of end product obtained in hexane.

4. Conclusion

Laccase derived from *R. vernificera* catalyzed the oxidative reaction of several phenolic compounds in organic solvent media. Laccase-catalyzed oxidation of (+)-catechin and (−)-epicatechin were enhanced in non-polar organic solvent with $\log p = 2.5–4.5$ rather than aqueous medium. The affinity between substrate and enzyme as well as catalysis efficiency were much higher with the substrates catechins than with catechol in the reaction media with $\log p = 3.5–4.5$ than those with $\log p = 2.0–2.5$. Laccase protein from *R. vernificera* was more efficient in hexane than in aqueous medium over a wide range of protein concentrations. The colorful end products from catechol oxidations were more stable than that formed in aqueous medium. All the results suggest that oxidation products from the phenolic compounds in organic solvent media are different from those obtained in aqueous medium. Further structure determination of the oxidation products is in progress.

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