Fluorometric assay of laccase in mushroom extracts and comparisons with absorption spectrophotometry

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ABSTRACT

Laccase is a lignin-degrading enzyme that is expected to move industrial applications to a greener form of biotechnology. Here, we used 2,2'-azinobis(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS) as a mediator and N-benzoyl leucomethylene blue (BLMB) as a substrate to develop a fluorometric assay that we used to measure laccase activity in mushroom extracts. We then compared this novel approach to conventional absorption spectrophotometry. With this novel approach, laccase oxidizes ABTS to produce ABTS radicals that show an absorption maximum at 415 nm. The ABTS radicals oxidize BLMB to generate fluorescent methylene blue that is measured by fluorometry while absorption spectrophotometry directly measures the absorbance of the ABTS radicals at 415 nm. Under the optimal conditions, the fluorometric assay showed a linear calibration curve with limits of detection and quantification of 1.0×10^{-2} mg mL^{-1} and 3.2×10^{-2} mg mL^{-1}, respectively, and those values are 1.4-fold lower than the results using conventional absorption spectrophotometry to measure ABTS radicals. Laccase activity of extracts from species of mushrooms that include eryngii and shiitake were successfully determined via both fluorometry and absorption spectrophotometry. The eryngii extract showed the highest level of activity, which was followed by the shiitake extract, but laccase activity was not observed in the shimeji extract.

1. Introduction

Laccase is a copper-containing polyphenol oxidase widely found in plants and fungi as well as in insects. Due to the ability of laccase to decompose aromatic polymers, it is expected to have utility in industrial fields for use in dye decolorization (Abadulla et al., 2000; Soares et al., 2001), wine clarification (Servili et al., 2000), and in bio-pulping (Dyer and Ragauskas, 2004). Another benefit is that the afore-mentioned processes, and others using laccase, can be performed under mild conditions. Compared with other lignin-degrading enzymes (lignin peroxidase and manganese peroxidase), laccase enables O_2 to replace H_2O_2 as the final electron acceptor, so the reaction is more economical and advantageous for industrial applications due to the lack of a need for additional H_2O_2. Laccase has a lower redox potential (0.5-0.8 V) than other enzymes (Munk et al., 2015) and can only catalyze the direct oxidation of para-diphenols, aminophenols, polyphenols, polyamines, and aryldiamines, as well as some inorganic ions (Giardina et al. 2010), but its enzymatic activity is expected to be useful in several industries.

Many mushrooms contain laccase, which produces vital life energy by decomposing cellulose and other organic compounds. For example, laccase is found in many edible mushrooms including *Cantharellus cibarius* (Ng and Wang, 2004), *Hypsizygus marmoreus* (Zhang et al., 2015), *Volvariella volvacea* (Chen et al., 2003), *Clitocybe maxima* (Zhang et al., 2010), *Pleurotus eryngii* (Wang and Ng, 2006b), and others (Ratcliffe et al., 1994). Some medicinal mushrooms contain laccase in their bodies as well (Ullrich et al., 2005; Wang and Ng, 2006a). Not only mushroom fruits but also mushroom compost (Matcham and Wood, 1992) and food industry waste (Songulashvili et al., 2006) are expected to be sources of laccase for industrial usage. Therefore, sensitive and reliable analytical methods are required for the assessment of laccase activity in the crude extracts of these sources.

The most conventional method of measuring laccase activity is absorption spectrophotometry, and a variety of chromogenic substrates that can be directly catalyzed

have been reported: 2,2′-azino-bis-3-ehtylbenzothiazoline-6-sulfonicacid (ABTS) (Bourbonnais and Paice, 1990), 2,6-dimethoxyphenol (Slomczynski et al., 1995), L-3,4-dihydroxyphenylalanine (Sinsabaugh et al., 1993), guaiacol (De Pinto and Ros Barceló, 1996), 4-methylcatechol (Shin et al., 1997), and syringaldazine (Harkin and Obst, 1973). The most widely used of these substrates is ABTS, because its sensitivity is three- to forty-fold higher than that of the other examples (Eichlerová et al. 2012). ABTS is also frequently used as an indicator of oxidants such as hydrogen peroxide (Cai et al., 2018), ozone (Wang and Reckhow, 2016), and peracetic acid (Yuan et al., 2022), and is employed in antioxidant tests (Teow et al., 2007; Martinez-Inda et al., 2023). However, Farnet et al. have reported that ABTS cannot realistically be considered as a relevant substrate for laccase activity, because in its oxidized form the ABTS free radicals ($ABTS^{\cdot+}$) are highly reactive, and abiotic reactions may occur during enzymatic measurement, which could lead to a misinterpretation of the results (Farnet et al., 2009; Farnet et al., 2011). Furthermore, absorption spectrophotometry could be interfered with if a sample contains colored components with the same wavelength as the absorption maximum of the ABTS free radicals.

To improve sensitivity and selectivity in the determination of laccase, fluorometry is an alternative because of potential advantages that include a small background signal, wide selectivity in excitation and emission wavelength, and no interference from colored substances. Thus, for the present study we developed a fluorometric assay of laccase to improve the sensitivity and selectivity. The fluorometric assay is based on the oxidation reaction of a fluorescent probe with the ABTS free radical that was generated by the enzymatic reaction of laccase. Laccase is known to react with ABTS to produce the ABTS radical, and then the ABTS radical oxidizes non-phenols (Bourbonnais and Paice, 1990). Therefore, a suitable fluorogenic substance could produce a usable fluorometric assay of laccase if it could be oxidized by the ABTS radical.

A few studies have demonstrated that N-benzoyl leucomethylene blue (BLMB) is a good probe for measurements of hydroperoxides and peroxidases via electrochemistry

(Pérusse and Leech, 2003) and surface-enhanced Raman spectroscopy (Guo et al., 2017). Some studies also reported that BLMB is a useful substrate for the enzymatic reaction of horseradish peroxidase (Ren and Kaneta, 2022; Danchana et al., 2022). In the present study, we found that the ABTS free radical, which is generated by a catalytic reaction with laccase, oxidizes BLMB to produce methylene blue that emits in the red region. The developed fluorometric assay was applied to an enzyme assay of mushroom extracts, and the results were compared with those of the ABTS-based absorption spectrophotometry.

2. Experimental

2.1 Chemicals

All chemicals were of analytical grade and were used without further purification. Deionized water was prepared by means of an Elix water purification system (Direct-Q UV3, Merck, Darmstadt, Germany). Laccase from *Trametes versicolor* was purchased from Merck (Darmstadt, Germany). BLMB was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methanol, Triton X-100, ABTS, sodium hydroxide, phosphoric acid, sodium dihydrogen phosphate, and disodium hydrogen phosphate were obtained from Wako Pure Chemical Corporation (Osaka, Japan). Acetic acid was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), and sodium acetate was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2 Sample preparation

Fresh mushroom samples, *Pleurotus eryngii* (eryngii mushroom), *Lentinula edodes* (shiitake mushroom), and *Hypsizygus marmoreus* (shimeji mushroom), were obtained from local supermarkets in Okayama city. Mushroom extracts were prepared as follows. Extraction of laccase from eryngii mushroom was accomplished using an established procedure reported in the literature (Wang and Ng, 2006b). A 40 g portion of eryngii mushroom was weighed and mixed with 20 mL of deionized water. The mixture was homogenized using a mortar and let stand overnight at 4 °C. The mixture was centrifuged

at 2,580 *g* for 15 minutes, and the supernatant was used as the eryngii extract. For shiitake mushrooms, a 20 g portion was gathered, mixed with 20 mL of water, homogenized using a mortar, let stand overnight at 4 °C, and centrifuged at 2,580 *g* for 15 minutes to produce the shiitake extract supernatant.

According to the literature (Teixeira et al., 2010), an extract of shimeji mushrooms was prepared by adding 20 mL of 0.02 M tartaric acid to a 20 g portion of shimeji mushrooms, which then was homogenized with a mortar, stored overnight in a refrigerator, and centrifuged at 2,580 *g* for 15 minutes. The resultant supernatant was used as the shimeji extract.

2.3 Apparatus

Absorption spectrophotometry and fluorometry were carried out using a UV–VIS spectrophotometer, UV2400-PC (Shimadzu, Kyoto, Japan), and a fluorometer, RF-5300PC (Shimadzu, Kyoto, Japan), respectively. The pH of the buffer solutions was measured via pH meter (Horiba, Kyoto, Japan).

2.4 Analytical methods

The pH values of the solutions were adjusted using H_3PO_4-NaH_2PO_4 (50 mM, pH 1.2-3.2), CH_3COOH-CH_3COONa (50 mM, pH 3.7-4.7), and NaH_2PO_4-Na_2HPO_4 (6.2-8.2). Standard solutions of laccase with concentrations of 5-25 mg mL^{-1} were prepared to construct absolute-calibration curves. In the absorption spectrophotometry, 100 µL of either standard or sample solutions were added to 10 mL of a buffer solution containing 5 µM ABTS. Absorbance at 415 nm was measured to quantify the produced ABTS radicals. In the fluorometric assay, the buffer solution contained 0.1(m/v)% Triton X-100 and 20 µM BLMB in addition to 5 µM of ABTS. To prepare the buffer solution, 25 mL of 500 mM phosphate buffer, 25 mL of 1(m/v)% Triton X-100, 12.5 mL of 100 µM ABTS, and 5 mL of 1 mM BLMB dissolved in methanol were added to a 250-mL volumetric

flask. The resulting solution was then diluted to a final volume of 250 mL with water. The concentration of BLMB was adjusted to 20 μM because it is insoluble in water and its solubility in 0.1% Triton X-100 solution is 20 μM as described in our previous study (Ren and Kaneta, 2022). The fluorescence of the produced methylene blue was measured at 650 nm and 680 nm for excitation and emission wavelengths, respectively. Crude extracts of mushrooms were mixed with the standard solutions with different concentrations at a ratio of 1:1 in the standard-addition methods of absorption spectrophotometry and fluorometry. A 100 μL aliquot of the mixture was added to 10 mL of the buffer solution containing 5 μM ABTS for absorption spectrophotometry and 20 μM BLMB in addition to 5 μM ABTS for fluorometry.

2.5 Statistical analysis

Microsoft Excel processed all of the data to calculate standard deviations, correlation coefficients, laccase concentrations, the limit of detection (LOD), the limit of quantification (LOQ), and recovery. Microsoft Excel was also used to construct the calibration curves. The standard deviations were calculated from three replicated measurements. The LOD and LOQ were calculated according to the International Union of Pure and Applied Chemistry (IUPAC) standards (Currie, 1999; Olivieri, 2015). Recoveries were evaluated using absolute calibration curves. Spiked and unspiked samples were measured by the absolute calibration methods, and the recovery was calculated by dividing the obtained concentration for the spiked sample by the sum of the obtained concentration for the unspiked sample and the spiked concentration.

3. Results and discussion

3.1 Absorption spectra of ABTS radicals and methylene blue

Laccase converts colorless ABTS to green ABTS radicals in the presence of oxygen. The absorption spectrum of the ABTS radicals generated by laccase and that of methylene

blue are shown in Fig. 1. In the 400 to 800 nm range, the ABTS radicals have an absorption maximum at 415 nm. On the other hand, the absorption spectrum of methylene blue overlaps that of the ABTS radicals. Therefore, it is difficult to measure methylene blue via absorption spectrophotometry in the presence of ABTS radicals. However, because the ABTS radicals are non-fluorescent, fluorometry permits selective measurement of methylene blue which emits in the region of 600-700 nm. Thus, fluorometry using BLMB as a substrate and ABTS as a mediator is expected to achieve a more sensitive laccase assay compared with that of absorption spectrophotometry.

3.2 Optimal pH

To optimize the pH of the enzyme assay, the pH values of the buffer solutions were varied from 1.8 to 7.8. The absorbance dependence on pH of ABTS radicals and that of the fluorescence of methylene blue appears in Fig. 2. The absorbance of the ABTS radicals was increased from pH 2 to 3, at which point the maximum was obtained. Further increase~~s~~ in pH showed a decrease in absorbance similar to previous reports of using ABTS (Palmieri et al., 1993; Afreen et al., 2017). Absorption spectrophotometry revealed that the optimal pH of the enzymatic reaction, indeed, was 3. Recent research that suggested an ideal pH range of 3-9 also supports this result (Rumpf et al., 2023).

When the ABTS radicals reacted with BLMB, a pH lower than 3 gave a higher fluorescence intensity for methylene blue, which showed that an acidic condition promoted the production of methylene blue (Fig. 2). However, the fluorescence intensity for a blank solution also increased in the acidic pH region (3.26 at pH 3 and 8.39 at pH 1), so the optimum pH was found to be 3, which is similar to that of absorption spectrophotometry.

3.3 Optimal concentration of ABTS

The ABTS concentrations were varied to 5, 10, and 15 μM under conditions where

the concentrations of BLMB and Triton X-100 were fixed at 20 µM and 0.1(w/w)%, respectively, which was the case in the previous report (Ren and Kaneta, 2022). Triton X-100 was needed to increase the solubility of BLMB in water. The results appear in Fig. 3 where the fluorescence intensity was plotted against time. It should be noted that the data from 0 to 3 min could not be available because it took roughly 3 min to mix solutions and set the cuvette in the fluorometer before each measurement. At an ABTS concentration of 5 µM, the fluorescence intensity was increased from 3 to 5 min at which point it became stable. Conversely, the fluorescence intensity was stable from 3-5 min at 10 µM ABTS, and then decreased after 5 min. For 15 µM ABTS, the fluorescence intensity was decreased from 3 to 10 min. The decreased fluorescence intensity at 15 µM ABTS could have been due to a decomposition of methylene blue caused by excess amounts of ABTS radicals. Recent studies demonstrated that ABTS degrades methylene blue (Asadi et al., 2020, Girelli et al. 2021). These results support the hypothesis that the decrease in fluorescence intensity would be due to the decomposition of the produced methylene blue by residual ABTS free radicals. With respect to reaction time and fluorescence intensity, 5 µM ABTS required an incubation time of longer than 5 min whereas 10 µM ABTS showed stable fluorescence intensity within 3-5 min. Also, the maximum fluorescence intensity of 10 µM ABTS was comparable to that of 5 µM ABTS. Therefore, 10 µM ABTS was chosen as the optimum concentration.

3.4 Quantitative performance

The quantitative performances of absorption spectrophotometry and fluorometry were evaluated using laccase standard solutions dissolved in water. Both methods showed good linearities with correlation coefficients of 0.9985 for spectrophotometry and 0.9954 for fluorometry. The limits of quantification were 4.4×10^{-2} mg mL^{-1} for absorption

spectrophotometry and 3.2×10^{-2} mg mL^{-1} for fluorometry. The fluorometric assay achieves a lower limit of quantification by a factor of 1.4.

3.5 Quantification of laccase activity in crude extracts of mushrooms

Under the optimal conditions, two methods were employed for the determination of crude extracts of eryngii, shiitake and shimeji mushrooms: absolute-calibration and standard-addition. Zhang et al. have reported that Hypsizygus marmoreus (bunashimeji) contained laccase (Zhang et al., 2015). However, in this study, laccase could not be found in the extract of shimeji mushrooms. Probably, the amount of laccase in the fruits of shimeji mushrooms is too small to be detected, which is different from bunashimeji. The definition of "shimeji" is ambiguous and several species of shimeji are found in markets. Thus, the content of laccase in shimeji mushrooms would depend on their species and cultivation environment.

The calibration curves in the measurements of the extract from eryngii mushrooms are shown in Fig. 4 where (a) and (b) were obtained by absorption spectrophotometry and fluorometry, respectively. The slopes of the standard-addition methods are in good agreement with those of the absolute-calibration curves for both absorption spectrophotometry and fluorometry. Correlation coefficients in fluorometry and absorption spectrophotometry are larger than 0.99 except for the standard-addition method of absorption spectrophotometry (See Fig. 4). The obtained contents of laccase in the eryngii extracts are summarized in Table 1. In absorption spectrophotometry, 0.375 and 0.325 mg g^{-1} were obtained via the absolute-calibration and standard-addition methods whereas fluorometry showed 0.230 mg g^{-1} and 0.270 mg g^{-1}. The results of absorption spectrophotometry and fluorometry seemed to show no significant differences, so the content of laccase in the fruits of eryngii mushrooms were found to be roughly 0.3 mg g^{-1} in both fluorometry and absorption spectrometry using both the absolute-calibration and standard-addition methods.

The calibration curves in the measurements of the extract from shiitake mushrooms

are shown in Fig. 5 and the obtained laccase activities are summarized in Table 1 as well. The absolute-calibration curves represented slightly different equations because the experiments were conducted on different days. Absorption spectrophotometry showed the same slope in both the absolute-calibration and the standard-addition methods. However, fluorometry showed a smaller slope in the results of the standard-addition method compared with that of absolute-calibration. The obtained laccase activities were 0.151 (absolute-calibration) and 0.130 (standard-addition) mg g^{-1} in absorption spectrophotometry whereas they were 0.066 (absolute-calibration) and 0.109 (standard-addition) mg g^{-1} in fluorometry. Consequently, estimates for the amount of laccase in the fruit of shiitake mushrooms is roughly 0.1 mg mL^{-1}.

The recoveries are also shown in Table 1. In the fluorometric assay, although the activity of the shiitake mushroom extract was slightly underestimated, the recoveries ranged from 86 to 108, which is acceptable in the measurement of real samples. In addition, in Table 1, all recoveries obtained by spectrophotometry and fluorometry were within 100±15% (85-115%), so the two methods showed no significant difference.

Although all values for the shiitake mushroom extract seemed to show no significant differences, the absolute calibration method by fluorometry possibly underestimated the amount of laccase, as evidenced by the difference in the slopes between the absolute-calibration and standard-addition methods. These facts suggest that the crude extract of shiitake mushrooms contains substances that inhibit the reaction between ABTS radicals and BLMB while no substance would show interference in the reaction between laccase and ABTS because of similar slopes between the absolute-calibration and the standard-addition methods in absorption spectrophotometry. Therefore, the fluorometric assay requires the standard-addition method to measure the crude extract of shiitake mushrooms in order to minimize the chances for underestimation. Conversely, the advantages of the fluorometric assay include low levels of limitations for quantification and for potential selectivity. The fluorometric assay would be useful for samples that contain colored substances that absorb light at 415 nm. For example, when the antioxidant test with ABTS

288 employs a pigment that has absorption at 415 nm, the antioxidant activity is
289 underestimated (Teow et al., 2007, Martinez-Inda et al., 2023). In such cases, the
290 fluorometric assay would resolve the issue of the absorption-spectrophotometric assay.
291 Therefore, the fluorometric assay could be an alternative to conventional absorption-
292 spectrophotometric assay.

293 To compare the activity of crude extracts with the previously reported values, the
294 activity was calculated using the international unit for enzyme which is defined as the
295 amount of enzyme that catalyzes the conversion of one micromole of substrate per minute.
296 The results are summarized in Table 2. In the listed literature where ABTS was used as
297 the substrate, the activity of laccase is defined as the amount of enzyme required to
298 produce an absorbance increase at 405 nm of one per minute per milliliter of reaction
299 mixture. Therefore, we converted these values to those expressed by the international unit
300 for enzyme using the absorption coefficient of ABTS radical as 3.6×10^4 $M^{-1}cm^{-1}$ (Childs
301 and Bardsley, 1975). It should be noted that the absorbance of APTS radial at 405 nm
302 seems to be similar to that at 415 nm. As seen in Table 2, the enzyme activities of the
303 mushroom extracts are comparable with the values reported in the literature. Extracts of
304 fresh mushrooms showed laccase activities lower than 1 U g^{-1} whereas recently studies
305 on fermented mushrooms exhibited extremely high activities (Rajavat et al., 2020;
306 Vaithyanathan et al., 2022). Therefore, the activities obtained in this study would be
307 reliable as supported by the literature.

308 The analytical performance of the developed fluorometric assay was also compared
309 with other methods reported in the literature as shown in Table 3. The LODs and LOQs
310 are lower than the other techniques such as the flow injection method, resonance
311 scattering method, and electrochemical method while the present method is comparable
312 with the fluorometric assay using Amplex Red. The advantage of the present method is
313 the short analysis time of 3 min compared to the fluorometric assay using Amplex Red
314 which needs 10 min for the reaction time. Thus, the present fluorometric assay can be an
315 alternate method to measure the laccase activity in crude mushroom extracts.

4. Conclusions

In this study, we developed a fluorometric assay for laccase using BLMB as the substrate and ABTS as the mediator and compared it with the results of conventional absorption spectrophotometry using ABTS. The fluorometric assay showed a good linear relationship with a 1.4-fold lower limit of quantification compared with conventional absorption spectrophotometry. Both methods were applied to measurements of laccase in crude extracts of mushroom samples, and the results of the fluorometric assay were comparable to those obtained via a conventional absorption-spectrophotometric assay, which suggests that the fluorometric assay accurately measures laccase activity in mushroom samples. The standard addition method is recommended for minimizing errors in the fluorometric assay because we found underestimation of laccase activity in the extract of shiitake mushrooms. The results indicate that eryngii mushrooms contain a larger amount of laccase than that found in either shimeji or shiitake mushrooms. The present research demonstrated that the fluorometric assay developed in this study would be complementary to conventional absorption spectrophotometry using ABTS for the assay of laccase activity when samples contain lower concentrations of laccase.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

References

Abadulla, E., Tzanov, T., Costa, S., Robra, K. H., Cavaco, P.A., Guebitz, G. M., 2000. Decolorization and detoxification of textile dyes with a laccase from Trametes hirsute. Appl. Environ. Microbiol., 66, 3357-3362. https://doi.org/10.1128/AEM.66.8.3357-3362.2000.

Afreen, S., Shamsi, T. N., Baig, M. A., Ahmad, N., Fatima, S., Qureshi, M. I., Hassan,

M. I., Fatma, T., 2017. A novel multicopper oxidase (laccase) from cyanobacteria: Purification, characterization with potential in the decolorization of anthraquinonic dye. PLoS One, 12, e0175144. https://doi.org/10.1371/journal.pone.0175144.

Asadi, E., Makhdoumi, A., Asoodeh, A., 2020. Laccase mediator system obtained from a marine spore exhibits decolorization potential in harsh environmental conditions. Ecotox. Environ. Safe., 191, 110184. https://doi-org-ssl.8611.top/10.1016/j.ecoenv.2020.110184.

Bourbonnais, R. and Paice, M. G., 1990. Oxidation of nonphenolic substrates – an expanded role for laccase in lignin biodegradation. FEBS Lett., 267, 99-102. https://doi.org/10.1016/0014-5793(90)80298-W.

Cai, H. H., Liu, X., Zou, J., Xiao, J. Y., Yuan, B. L., Li, F., Cheng, Q. F., 2018. Multi-wavelength spectrophotometric determination of hydrogen peroxide in water with peroxidase-catalyzed oxidation of ABTS. Chemosphere, 193, 833-839. https://doi-org-ssl.8611.top/10.1016/j.chemosphere.2017.11.091.

Chen, S., Ma, D., Ge, W., Buswell, J. A., 2003. Induction of laccase activity in the edible straw mushroom, Volvariella volvacea. FEMS Microbiol. Lett., 218,143-148. https://doi.org/10.1111/j.1574-6968.2003.tb11510.x.

Childs, R. E. and Bardsley, W. G., 1975. The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem. J., 145, 93–103. https://doi.org/10.1042/bj1450093.

Cuadrado, M. U., Perez-Juan, P. M., de Castro, M. D. L., Gomez-Nieto, M. A., 2005. A fully automated method for in real time determination of laccase activity in wines. Anal. Chim. Acta, 553, 99-104. https://doi.org/10.1016/j.aca.2005.07.010.

Currie, L.A., 1999. Nomenclature in evaluation of analytical methods including detection and quantification capabilities[1] (IUPAC Recommendations 1995). Anal. Chim. Acta 391, 105-126. https://doi.org/10.1016/S0003-2670(99)00104-X.

Danchana, K., Iwasaki, H., Ochiai, K., Namba, H., Kaneta, T., 2022. Determination of glutamate using paper-based microfluidic devices with colorimetric detection for

372 food samples. Microchem J., 179, 107513,
373 https://doi.org/10.1016/j.microc.2022.107513.
374 De Pinto, M. C. and Ros Barceló, A., 1996. Inhibition of both peroxidase and laccase by
375 desferal (desferrioxamine mesylate). Phytochem., 42, 283-286.
376 https://doi.org/10.1016/0031-9422(95)00984-1.
377 Dyer, T. J. and Ragauskas, A. J., 2004. Laccase: a harbinger to kraft pulping. ACS
378 Symposium Series, 889, 339-362. https://doi.org/10.1021/bk-2004-0889.ch020.
379 Eichlerová, I., Šnajdr, J., Baldrian, P., 2012. Laccase activity in soils: Considerations for
380 the measurement of enzyme activity. Chemosphere, 88, 1154-1160.
381 https://doi.org/10.1016/j.chemosphere.2012.03.019.
382 Farnet, A. M., Chevremont, A. C., Gil, G., Gastaldi, S., Ferré, E., 2011. Alkylphenol
383 oxidation with a laccase from a white-rot fungus: Effects of culture induction and of
384 ABTS used as a mediator. Chemosphere, 82, 284-289.
385 https://doi.org/10.1016/j.chemosphere.2010.10.001.
386 Farnet, A. M., Gil, G., Ruaudel, F., Chevremont, A. C., Ferre E., 2009. Polycyclic
387 aromatic hydrocarbon transformation with laccases of a white-rot fungus isolated
388 from a Mediterranean schlerophyllous litter. Geoderma, 149, 267-271.
389 https://doi.org/10.1016/j.geoderma.2008.12.011.
390 Gáspár, S., Brinduse E., Vasilescu A., 2020. Electrochemical evaluation of laccase
391 activity in must. Chemosensors 8, 126.
392 https://doi.org/10.3390/chemosensors8040126
393 Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., Sannia, G., 2010.
394 Laccases: a never-ending story. Cell. Mol. Life Sci., 67, 369–385.
395 https://doi.org/10.1007/s00018-009-0169-1.
396 Girelli, A. M., Pambianco, E., Scuto, F. R., 2021. Sustainable recycling of spent grain
397 for laccase immobilization as dyes removal tool. J. Environ. Chem. Eng., 9, 106653.
398 https://doi.org/10.1016/j.jece.2021.106653.
399 Guo, W., Hu, Y., Wei, H., 2017. Enzymatically activated reduction-caged SERS

reporters for versatile bioassays. Analyst, 142, 2322 -2326. https://doi.org/10.1039/c7an00552k.

Harkin, J. M. and Obst, J. R., 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. Experientia, 29, 382-387. https://doi.org/10.1007/bf01926734.

Jiang, C. N., Ling, S. M., Wang, P. F., Liang, A. H., Chen, B., Wen, G. Q., Jiang, Z. L., 2011. A new and sensitive catalytic resonance scattering spectral assay for the detection of laccase using guaiacol as substrate. Luminescence., 26, 500-505. https://doi.org/10.1002/bio.1259.

Liang, A. H., Wang, P. F., Jiang, Z. L., 2011. A New and Sensitive Catalytic Resonance Scattering Spectral Assay for the Detection of Laccase Activity Using H2O2-I⁻-TDMAC System. Chin. J. Chem., 29, 787-792. https://doi.org/10.1002/cjoc.201190159.

Martinez-Inda, B., Esparza, I., Moler, J. A., Jimenez-Moreno, N., Ancin-Azpilicueta, C., 2023. Valorization of agri-food waste through the extraction of bioactive molecules. Prediction of their sunscreen action. J. Environ. Manage., 325, 116460. https://doi.org/10.1016/j.jenvman.2022.116460.

Matcham, S. E. and Wood, D. A., 1992. Purification of Agaricus bisporus extracellular laccase from mushroom compost. Biotechnol. Lett., 14, 297-300. https://doi.org/10.1007/BF01022327.

Munk, L., Sitarz, A. K., Kalyani, D. C., Dalgaard Mikkelsen, J., Meyer, A.S., 2015. Can laccases catalyze bond cleavage in lignin?. Biotechnol. Adv., 33, 13-24. https://doi.org/10.1016/j.biotechadv.2014.12.008.

Ng, T. B. and Wang, H. X., 2004. A homodimeric laccase with unique characteristics from the yellow mushroom Cantharellus cibarius. Biochem. Biophys. Res. Commun., 313, 37-41. https://doi.org/10.1016/j.bbrc.2003.11.087.

Olivieri, A. C., 2015. Practical guidelines for reporting results in single- and multi-component analytical calibration: A tutorial. Anal. Chim. Acta, 868, 10–22.

https://doi.org/10.1016/j.aca.2015.01.017.
Palmieri, G., Giardina, P., Marzullo, L., Desiderio, B., Nitti, G., Cannio, R., Sannia.G., 1993. Stability and activity of a phenol oxidase from the ligninolytic fungus Pleurotus ostreatus. Appl. Microbiol. Biotechnol.. 39, 632-636. https://doi.org/10.1007/BF00205066.
Pérusse, P. and Leech, D., 2003. A voltammetric assay of antioxidants and inhibitors of soybean lipoxygenase. Electroanalysis, 15, 573-578. https://doi.org/10.1002/elan.200390071.
Rajavat, A. S., Rai, S., Pandiyan, K., Kushwaha, P., Choudhary, P., Kumar, M., Chakdar, H., Singh, A., Karthikeyan, N., Bagul, S. Y., Agnihotri, A., Saxena, A. K., 2020. Sustainable use of the spent mushroom substrate of Pleurotus florida for production of lignocellulolytic enzymes. J. Basic Microbiol., 60, 173-184. https://doi.org/10.1002/jobm.201900382.
Ratcliffe, B., Flurkey, W. H., Kuglin, J., Dawley, R., 1994. Tyrosinase, lactase, and peroxidase in mushrooms (agaricus, crimini, oyster, and shiitake). J. Food Sci., 59, 824-827. https://doi.org/10.1111/j.1365-2621.1994.tb08137.x.
Ren, J. and Kaneta, T., 2022. N-Benzoyl leucomethylene blue as a novel substrate for the assays of horseradish peroxidase by spectrophotometry and capillary electrophoresis-laser-induced fluorometry. Anal. Sci., 38, 651-655. https://doi.org/10.1007/s44211-022-00078-7.
Servili, M., DeStefano, G., Piacquadio, P., Sciancalepore, V., 2000. A novel method for removing phenols from grape must. Am. J. Enol. Vitic., 51, 357-361. https://doi.org/10.5344/ajev.2000.51.4.357.
Shin, R., Froderman, T., Flurkey, W. H., 1997. Isolation and characterization of a mung bean leaf polyphenol oxidase. Phytochemistry, 45, 15-21. https://doi.org/10.1016/S0031-9422(96)00785-6.
Sinsabaugh, R. L., Antibus, R. K., Linkins, A. E., McClaugherty, C. A., Rayburn, L., Repert, D., Weiland, T., 1993. Wood decomposition – nitrogen and phosphorus

456 dynamics in relation to extracellular enzyme activity. Ecology, 74, 1586-1593.
457 https://doi.org/10.2307/1940086.
458 Slomczynski, D., Nakas, J. P., Tanenbaum, S. W., 1995. Production and characterization
459 of laccase from Botrytis cinerea 61–34. Appl. Environ. Microbiol., 61, 907-912.
460 https://doi.org/10.1128/AEM.61.3.907-912.1995.
461 Soares, G. M. B., Costa, F. M., De Amorium, M. T. P., 2001. Decolorization of an
462 anthraquinone-type dye using a laccase formulation. Bioresour. Technol., 79, 171-
463 177. https://doi.org/10.1016/S0960-8524(01)00043-8.
464 Songulashvili, G., Elisashvili, V., Wasser, S., Nevo, E., Hadar, Y., 2006. Laccase and
465 manganese peroxidase activities of Phellinus robustus and Ganoderma adspersum
466 grown on food industry wastes in submerged fermentation. Biotechnol. Lett., 28,
467 1425-1429. https://doi.org/10.1007/s10529-006-9109-4.
468 Teixeira, R. S., Pereira, P. M., Ferreira-Leitão, V. S., 2010. Extraction and application of
469 laccases from shimeji mushrooms (Pleurotus ostreatus) residues in decolourisation
470 of reactive dyes and a comparative study using commercial laccase from Aspergillus
471 oryzae. Enzyme Research, 2010, 905896. https://doi.org/10.4061/2010/905896.
472 Teow, C.C., Truong, V. D., McFeeters, R. F., Thompson, R. L., Pecota, K. V., Yencho,
473 G. C., 2007. Antioxidant activities, phenolic and β-carotene contents of sweet potato
474 genotypes with varying flesh colours. Food Chem., 103, 829-838.
475 https://doi.org/10.1016/j.foodchem.2006.09.033.
476 Ullrich R., Huong L. M., Dung, N. L., Hofrichter, M., 2005. Laccase from the medicinal
477 mushroom Agaricus blazei: production, purification and characterization. Appl.
478 Microbiol. Biotechnol., 67, 357-363. https://doi.org/10.1007/s00253-004-1861-6.
479 Vaithyanathan, V. K., Vaidyanathan, V. K., Cabana, H., 2022. Laccase-Driven
480 Transformation of High Priority Pesticides Without Redox Mediators: Towards
481 Bioremediation of Contaminated Wastewaters. Front. Bioeng. Biotechnol., 9,
482 770435. https://doi.org/10.3389/fbioe.2021.770435.
483 Wang, H. X. and Ng, T. B., 2006a. A laccase from the medicinal mushroom Ganoderma

lucidum. Appl. Microbiol. Biotechnol.. 72, 508-513. https://doi.org/10.1007/s00253-006-0314-9.

Wang, H. X. and Ng, T. B., 2006b. Purification of a laccase from fruiting bodies of the mushroom Pleurotus eryngii. Appl. Microbiol. Biotechnol., 69, 521-525. https://doi.org/10.1007/s00253-005-0086-7.

Wang, T., Xiang, Y. Q., Liu, X. X., Chen, W. L., Hu, Y. G. 2017. A novel fluorimetric method for laccase activities measurement using Amplex Red as substrate. Talanta, 162, 143-150. https://doi.org/10.1016/j.talanta.2016.10.006.

Wang, T., and Reckhow, D.A., 2016. Spectrophotometric method for determination of ozone residual in water using ABTS: 2.2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonate). Ozone Sci. Eng., 38, 373-383. https://doi.org/10.1080/01919512.2016.1188681.

Yuan, N., Li, H. C., Qian, J. S., 2022. Determination of peracetic acid in the presence of hydrogen peroxide based on the catalytic oxidation of ABTS. Chem. Eng. J. Adv. 10, 100247. https://doi-org-ssl.8611.top/10.1016/j.ceja.2022.100247.

Zhang, G.-Q., Wang, Y.-F., Zhang, X.-Q., Ng, T. B., Wang, H.-X., 2010. Purification and characterization of a novel laccase from the edible mushroom Clitocybe maxima, Process Biochem., 45, 627-633. https://doi.org/10.1016/j.procbio.2009.12.010.

Zhang, J., Chen, H., Chen, M., Ren, A., Huang, J., Wang, H., Zhao, M., Feng, Z., 2015. Cloning and functional analysis of a laccase gene during fruiting body formation in Hypsizygus marmoreus. Microbiol. Res., 179, 54-63. https://doi.org/10.1016/j.micres.2015.06.005.

Zou, Y. J., Wang, H. X., Ng, T. B., Huang, C. Y., Zhang J. X., 2012. Purification and characterization of a novel laccase from the edible mushroom Hericium coralloides. J. Microbiol., 50, 72–78. https://doi.org/10.1007/s12275-012-1372-6.

Table 1. Determination of laccase in crude mushroom extracts.

Extraction source	Laccase in mushroom					
	Absorption spectrophotometry			Fluorometry		
	Absolute calibration method/ mg g^{-1}	Standard addition method/ mg g^{-1}	Recovery/ %	Absolute calibration method/ mg g^{-1}	Standard addition method/ mg g^{-1}	Recovery */ %
Eryngii mushroom	0.373~~5~~± 0.009	0.327~~5~~± 0.004	95-111[a]	0.229~~30~~ ±0.016	0.270± 0.013	103-111[b]
Shiitake mushroom	0.151± 0.006	0.130± 0.015	94-108[c]	0.066± 0.001	0.109± 0.001	86-108[d]

Concentrations of the standard in the samples: a, 0.05, 0.10, 0.15, and 0.20 mg mL^{-1}; b, 0.025, 0.05, 0.10, and 0.15 mg mL^{-1}; c, 0.025, 0.05, 0.10, and 0.15 mg mL^{-1}; d, 0.025, 0.05, 0.075, and 0.10 mg mL^{-1}.

*Recovery values were obtained by the absolute calibration methods.

519 Table 2. Comparison of laccase activity in mushrooms

Mushroom	Activity/ U g^{-1}	Activity calculated from the international unit for enzyme**/ U g^{-1}	References
Clitocybe maxima	7.06*	0.3	Zhang et al., 2010
Cantharellus cibarius	2.3*	0.08	Ng and Wang, 2004
Hericium coralloides	2.95*	0.1	Zou et al., 2012
Pleurotus eryngii	9.0*	0.3	Wang and Ng, 2006b
Ganoderma lucidum	2.5*	0.09	Wang and Ng, 2006a
Pleurotus florida (fermentation)	3,015.8	3,015.8	Rajavat et al., 2020
Pleurotus dryinus (fermentation)	162.1	162.1	Vaithyanathan et al., 2022
Shiitake mushroom	0.12 (spectrophotometry)	0.3	This work
	0.0981 (Fluorimetry)	N/A	
Pleurotus eryngii	0.29 (spectrophotometry)	0.6	
	0.24 (Fluorimetry)	N/A	

520 * One unit of enzyme activity was defined as the amount of enzyme required to produce
521 an absorbance increase at 405 nm of one per minute per milliliter of reaction mixture.
522 ** One unit of enzyme activity was defined as the amount of enzyme that catalyzes the
523 conversion of one micromole of substrate (ABTS) per minute.
524 N/A: Not applicable
525

526 Table 3. Comparison of analytical performance with the reported methods.

Method	Substrate (and mediator)	LOD / U mL^{-1}	LOQ / U mL^{-1}	Analysis time / min	References
Flow injection spectrophotometry	Syringaldazine	0.2	0.6	12	Cuadrado et al., 2005
Resonance scattering	Guaiacol	0.05	N/A	35	Jiang et al., 2011
Resonance Scattering	NaI, H_2O_2, tetradecyl timethylbenzyl ammonium chloride	0.02	N/A	10	Liang et al., 2011
Fluorimetry	Amplex Red	1.76×10^{-3}	N/A	10	Wang et al., 2017
Electrochemistry	Syringaldazine, ABTS	0.5 U	N/A	N/A	Gáspár et al., 2020.
Fluorimetry	ABTS and BLMB	9×10^{-3}	2.9×10^{-2}	3	This work

527 N/A: Not applicable

528

529 **Figure Captions**

530 Fig. 1. Absorption spectra of ABTS radicals and methylene blue. ABTS free radicals were
531 formed by incubation of 0.5 mM ABTS solution in phosphate buffer with 0.5 mg mL^{-1} of
532 laccase (10 mL of 0.5 mg mL^{-1}). The concentration of methylene blue, 10 μM.

533

534 Fig. 2. Effect of pH on fluorometry using BLMB as a substrate. The pH was adjusted
535 using phosphate and acetate buffers. Sample: 0.5 mg mL^{-1} of laccase in 50 mM buffer
536 containing 0.10% (m/v) Triton X-100, 10 μM ABTS, and 20 μM BLMB. Reaction time,
537 2 min. The absorbance of ABTS radicals was measured at 415 nm. The fluorescence of
538 methylene blue was measured at an excitation wavelength of 650 nm and at an emission
539 wavelength of 680 nm.

540

541 Fig. 3. Effect of ABTS concentration on the reaction with BLMB. Sample: 0.5 mg mL^{-1}
542 of laccase in 50 mM phosphate buffer (pH 3) containing 0.10% (m/v) Triton X-100,
543 different concentrations of ABTS (5, 10, and 15 μM), and 20 μM BLMB. The
544 fluorescence of methylene blue was measured at an excitation wavelength of 650 nm and
545 at an emission wavelength of 680 nm.

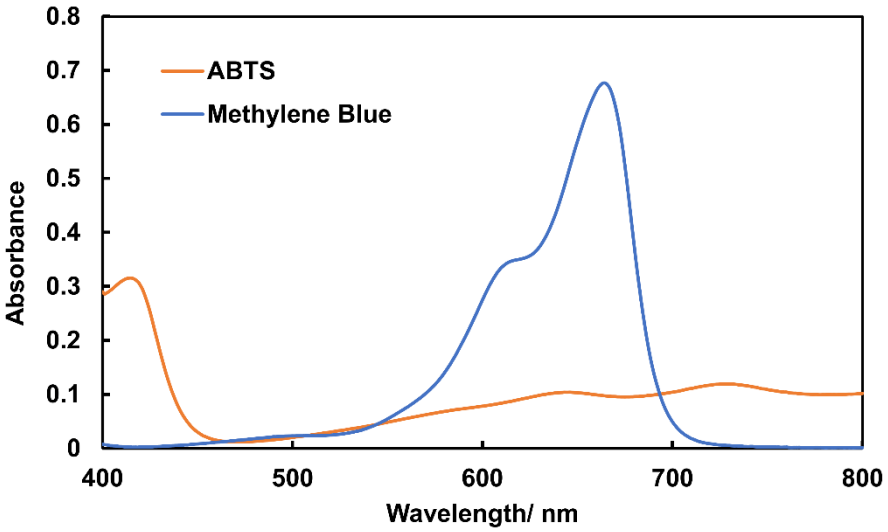
546

547 Fig, 4. Calibration curves obtained by absolute-calibration and standard-addition methods
548 for the determination of an eryngii extract. Red line, absolute-calibration method; blue
549 line, standard-addition method. (a) absorption spectrophotometry, (b) fluorometry.
550 Sample, eryngii extract. Conditions are described in the text.

551

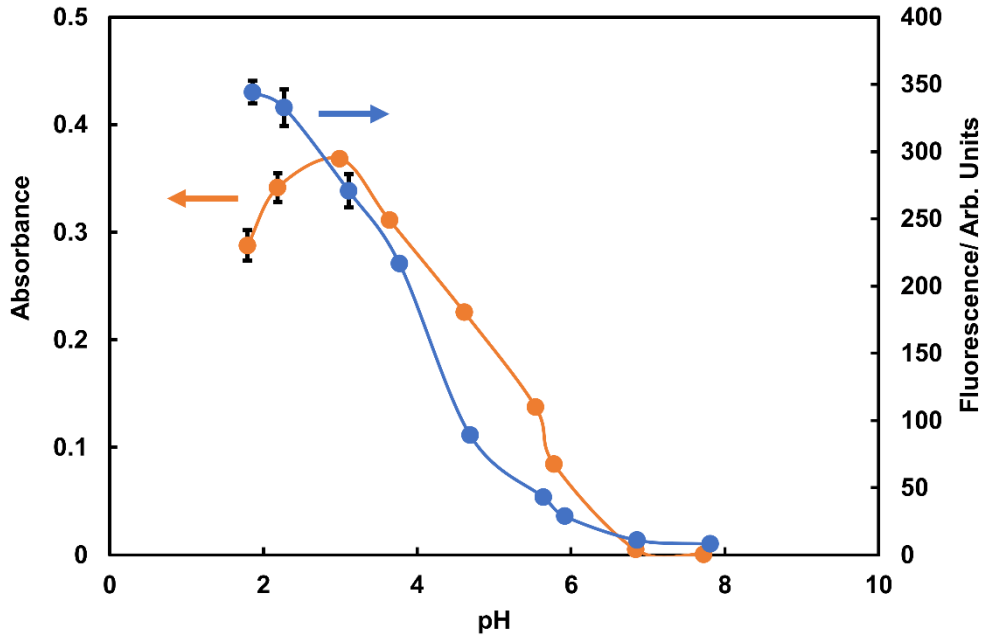
552 Fig, 5. Calibration curves obtained by absolute-calibration and standard-addition methods
553 for the determination of a shiitake extract. Red line, absolute-calibration method; blue
554 line, standard-addition method. (a) absorption spectrophotometry, (b) fluorometry.
555 Sample, shiitake extract. Conditions are described in the text.

556


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
ABTS
Methylene Blue
400
500
600
700
800
Wavelength/ nm

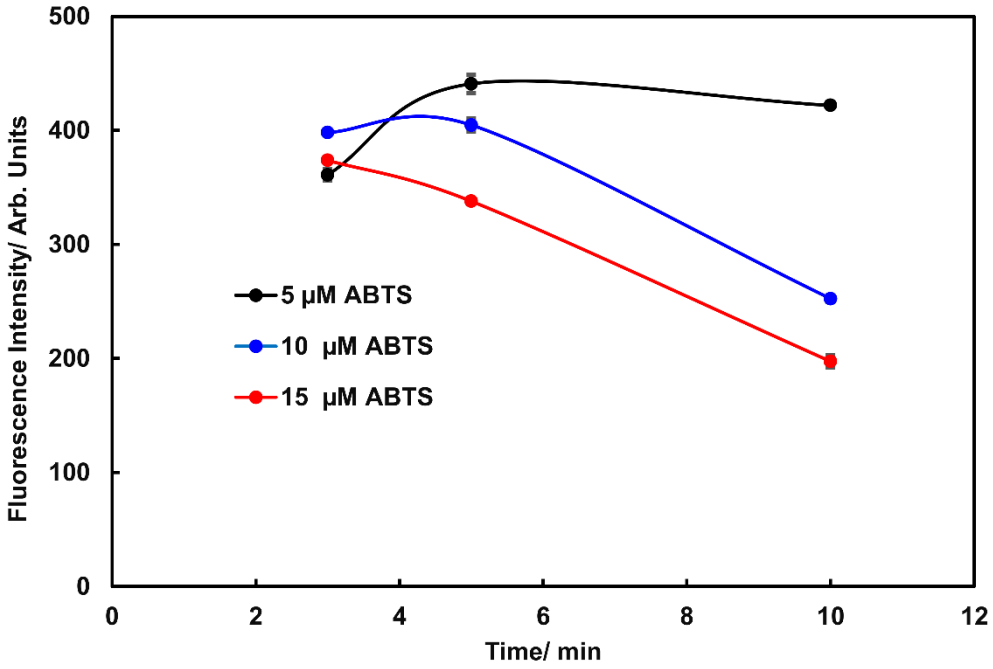
557

558 Fig. 1


0.5
0.4
0.3
0.2
0.1
0
Absorbance
400
350
300
250
200
150
100
50
0
Fluorescence/ Arb. Units
0
2
4
6
8
10
pH

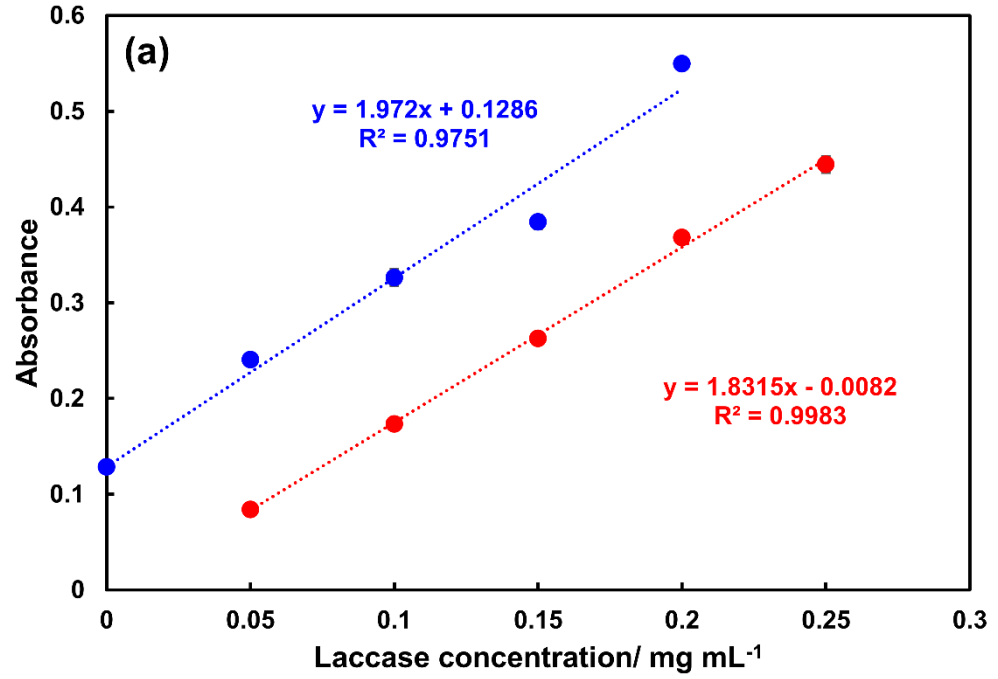
559

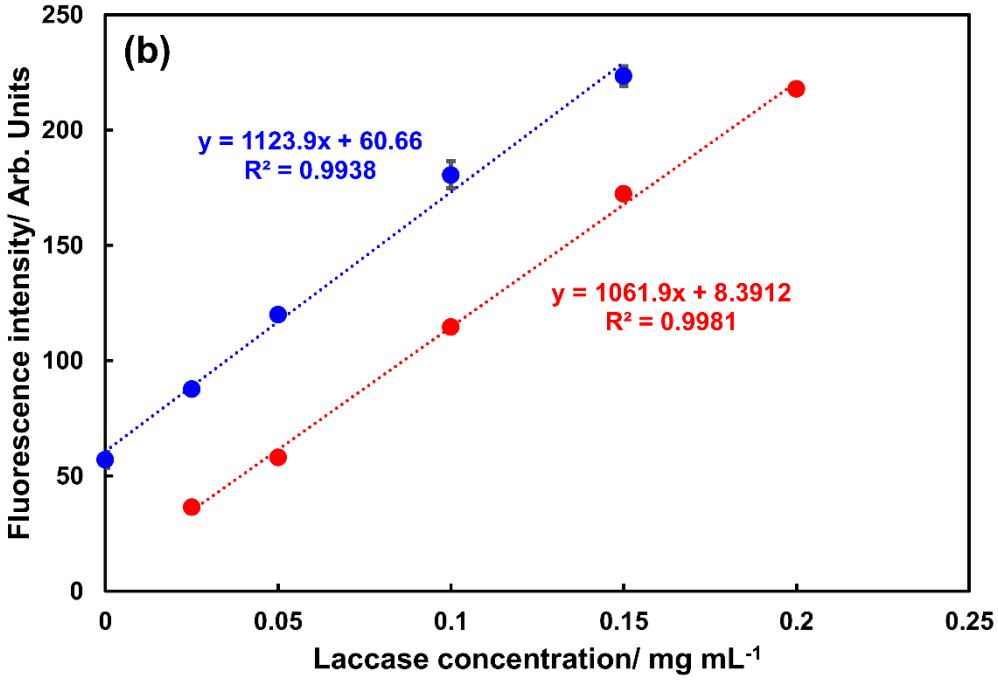
560 Fig. 2


Fluorescence Intensity/ Arb. Units
Time/ min
5 μM ABTS
10 μM ABTS
15 μM ABTS
0
100
200
300
400
500
0
2
4
6
8
10
12

561

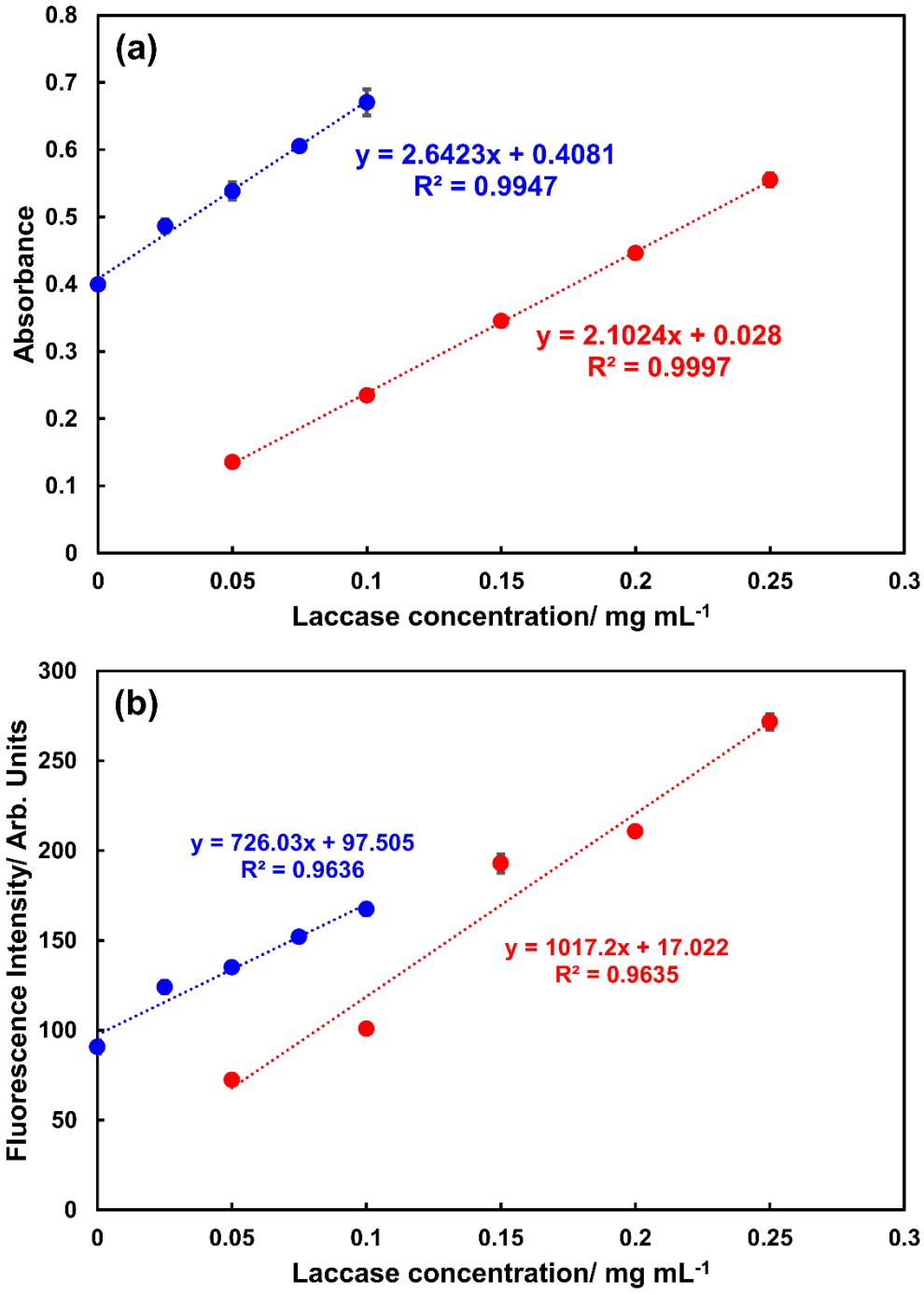
562 Fig. 3


(a)
y = 1.972x + 0.1286
R² = 0.9751
y = 1.8315x - 0.0082
R² = 0.9983
Absorbance
Laccase concentration/ mg mL-1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.05
0.15
0.25


(b)
y = 1123.9x + 60.66
R² = 0.9938
y = 1061.9x + 8.3912
R² = 0.9981
Fluorescence intensity/ Arb. Units
Laccase concentration/ mg mL-1
0
50
100
150
200
250
0.05
0.1
0.15
0.2
0.25

563

564 Fig. 4


(a)
y = 2.6423x + 0.4081
R² = 0.9947
y = 2.1024x + 0.028
R² = 0.9997
Absorbance
Laccase concentration/ mg mL-1
(b)
y = 726.03x + 97.505
R² = 0.9636
y = 1017.2x + 17.022
R² = 0.9635
Fluorescence Intensity/ Arb. Units
Laccase concentration/ mg mL-1

565

566 Fig. 5