CHAPTER 5

PURIFICATION, CHARACTERIZATION AND SOME PROPERTIES OF LACCASE FROM *Trametes polyzona* WR710-1

5.1 Introduction

Laccase (EC 1.10.3.2) is an important enzyme in fungal ligninolytic systems involved in lignin degradation (Gianfreda *et al*., 1999). This enzyme, belonging to a family of multicopper-containing enzymes, catalyzes the oxidation of a variety of phenolic compounds, polyamines, aryl diamines, and lignin, with concomitant reduction of O_2 to water (Thurston, 1994; Solomon *et al*., 1996). Most laccases were reported and studied from fungal organisms such as *Coriolus versicolor* (Madhavi and Lale, 2006), *Phanerochaete chrysosporium* (Elisashvili *et al*., 2008) and *Coriolopsis* (*Trametes*) *polyzona* (Jaouani *et al*., 2006). The purified fungal laccase are normally blue, copper-containing, proteins capable of oxidizing *o*- and *p*- phenols and aromatic amines and different substrates based on their substrate specificity (Hewitt and Smith, 1975). The different properties of purified laccase from various white-rot fungi have been reported (Lee *et al*., 1999; Saparrat *et al*., 2002; and Patrick *et al*., 2009). Some report found more than one isozyme produced from fungi such as, *Pleurotus ostreatus* V-184 produced four laccase isozymes (Mansur *et al*., 2003).

There are several applications of laccase enzyme in many fields such as, the pulp and paper industry (Bajpai, 1999), and food industries (Durán *et al*., 2002). Moreover, some properties of laccase was the enzyme having high potential for

environmental detoxification, because some fungal laccases have been reported for bisphenol A degradation (Fukuda *et al*., 2001; Michizoe *et al*., 2005 and Alessandro *et al*., 2008) and dye decolorization (Cameselle *et al*., 2003; Li *et al*., 2009 and Rim *et al*., 2010).

Bisphenol A; 2,2-bis(4-hydroxyphenyl) propane is a compound that is used as a material for synthesis of polycarbonate and epoxy plastic. Those are used widely in food and beverage containers, dental materials, and lacquers (Kuch and Ballschmiter, 2001). The contamination of bisphenol A into environment can cause for serious environmental concerns, because bisphenol A significantly affects on human and animal health (Alessandro *et al*., 2008). Recently, Chai *et al*. (2005) reported that four fungal strains, *Aspergillus terreus* MT-3, *Fusarium mornilforme* 2-2, *F. sporotrichioides* NFRI-1012, and *Emericella nidulans* MT-98 were effectively degrade of bisphenol A. Moreover, several basidiomycetous laccases, *Trametes villosa* (Fukuda *et al*., 2001), *T. versicolor* and *Polyporus pinisitus* (Claus *et al*., 2002) and *Grifola frondosa* (Nitheranont *et al*., 2011) revealed the potential of bisphenol A degradation.

Synthetic dyes are widely used in several industries including textile, paper printing, food processing, cosmetics and pharmaceuticals. These compounds are aromatic, water-soluble, dispersible organic colorants classified as azo, anthraquinone, heterocyclic, triphenylmethane or phthalocyanine dyes (Kandelbauer and Güebitz, 2004). It is estimated that 10-15% of the dyes are lost in the effluent during dyeing process (Laura *et al*., 2010). The dyes are released into environment and cause serious environmental problem and they are not easily degraded by the conventional wastewater treatments such as activated sludge and trickling filter etc.

(Shaul *et al*., 1991). White-rot fungi have attracted increasing attention as their ligninolytic enzymes, especially laccase, have ability to degrade recalcitrant compounds and synthetic dyes. In recent years, white-rot fungi such as *Pleurotus ostreatus* (Hongman *et al*., 2004), *Rigidoporus lignosus* W1 (Li *et al*., 2009), *Trametes trogii*, *T villosa* and *Coriolus versicolor* (Laura *et al*., 2010) were shown to have ability to decolorize different synthetic dyes. However, there are a few reports on the bisphenol A degradation and synthetic dyes decolorization by laccase from *T. polyzona*. Thus, in this experiment, purified laccase from *T. polyzona* WR710-1 was studied for the first time on its ability to degrade bisphenol A and dye decolorization.

In this study, we used the white-rot basidiomycetous fungus *Trametes polyzona* WR710-1 for production of laccase in solid state cultivation by using orange peel as substrate. Many species of *Trametes* have been reported as laccase producers for example, *T. versicolor* (Jing *et al*., 2007; Tišma *et al*., 2012), *T. hirsute* (Rosales *et al*., 2002; Couto *et al.,* 2006) and *T. trogii* (Héla *et al*., 2006; Kocyigit *et al*., 2012). Although there are some previous studies on laccase production from *T. polyzona*, however steps for purification and characterization of this enzyme have not been studied so far. In addition, there are a few reports on some properties of laccase from *T. polyzona* such as bisphenol A degradation and synthetic dyes decolorization. Thus in this experiment, laccase from *T. polyzona* WR710-1 was purified, charaterized and some detoxification properties was studied.

5.2 Materials and methods

5.2.1 Organism and culture conditions

According to the optimal condition for laccase production (chapter 4), cultures were incubated at 37 ^{o}C for 14 days. Crude enzymes were extracted by adding 100 ml of 20 mM potassium phosphate buffer pH 7.0 (buffer A) to the culture and mixed at 4 ^{o}C for 1 hour. The culture extract was filtered through a cotton cloth and centrifuged at 8,000 rpm for 15 min at 4 ^{o}C. The obtained supernatant was used as an enzyme solution.

5.2.2 Purification of laccase

All purification steps were carried out at 4 ^{o}C. Ammonium sulfate was added to the culture extract prepared as mentioned above to achieve 30 % saturation and then applied to a Toyopearl Buttyl-650 M column (2.5×25 cm, Tosoh, Tokyo) equilibrated with buffer A containing 30 % saturated ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate (400 ml, 30-0 % saturation) in buffer A at a flow rate of 1.0 ml/min. The main fraction containing laccase activity was collected and dialyzed against buffer A. The resulting solution was applied to a HiPrep 16/10 Q XL column (GE Healthcare, Buckinghamshire, UK) equipped with an ÄKTA prime liquid chromatographic system (GE Healthcare). The enzyme was eluted with a linear gradient of NaCl (400 ml, 0-0.5 M) in buffer A at a flow rate of 1.0 ml/min. Fractions having laccase activity were dialyzed. Ammonium sulfate was added to achieve 30 % saturation in the enzyme solution. The solution was applied to a HiLoad 16/60 Phenyl Sepharose HP column (GE Healthcare), and the enzyme was eluted with a linear gradient of ammonium sulfate (400 ml, 30-0 % saturation) in buffer A at a flow rate of 1.0 ml/min. The active fractions were dialyzed. The sample was applied to a

HiPrep 16/10 Q XL column under the same conditions mentioned above. The active fraction was dialyzed, concentrated using a 10 kDa molecular weight cut-off ultrafiltration concentrator (Centriprep-10, Millipore, Bradford, MA), and then applied to a HiLoad 16/60 Superdex 200 pg column (GE Healthcare). The enzyme was eluted with buffer A at a flow rate of 1.0 ml/min. The active fraction was used for further experiments. The purity of the enzyme was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.3 Molecular mass determination

The apparent molecular mass determination was carried out by gel filtration and SDS-PAGE. Gel filtration was done with a HiLoad 16/60 Superdex 200 g. Cytochrome C (12.4 kDa), carbonic anhydrate (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa) (Sigma, St. Louis, MO) were used to calibrate the column. SDS-PAGE was carried out according to the procedure of Laemmli (1970) with marker proteins from 250-10 kDa (XL-ladder broad; APRO Life Science Institute, Tokushima, Japan).

5.2.4 Absorption spectrum

The absorption spectrum was recorded between 250 and 700 nm using a U-2010 spectrophotometer (Hitachi, Tokyo) with a 1.0 cm light path. The absorption spectrum was measured in 20 mM potassium phosphate buffer (pH 7.0) at an enzyme concentration of 0.73 mg/ml.

5.2.5 Substrate specificity and kinetic parameters

In order to examine the catalytic properties of the purified laccase, the Michaelis constant (K_m), the maximum reaction velocity (V_{max}), and the catalytic constant (k_{cat}) for five different substrates 0.02-0.6 mM of 2,6-DMP in sodium acetate buffer (buffer

B) pH 5.0, 0.5-3 mM of guaiacol in buffer B pH 4.0, 1-4 mM of catechol in buffer B pH 4.0, 1-4 mM of L-DOPA in buffer B pH 4.0 and 0.05-1.2 mM of ABTS in sodium tartrate buffer pH 2.0 were used. The assay mixture contained a substrate, 100 mM of buffer, and enzyme in a total volume of 1 ml and was determined spectrophotometrically at 30^{o}C for 2 min per reaction.

5.2.6 Effects of pH and temperature

To determine the effects of pH and temperature on laccase activity and stability, purified laccase was used. The optimum pH was measured with the five substrates mentioned before. The pH in the reaction mixture was adjusted between 2.0-6.0 using 100 mM sodium tartrate buffer and 100 mM sodium acetate buffer. To determine the effect of pH on enzyme stability, purified laccase was incubated at 28 ^{o}C for 24 h in various buffers: 100 mM sodium tartrate buffer (pH 2.0-3.0), 100 mM sodium acetate buffer (pH 3.0-6.0), 100 mM potassium phosphate buffer (pH 6.0-8.0) and 100 mM Tris-SO_4 buffer (pH 8.0-10.0) containing 1 µg ml^{-1} BSA. Then the remaining enzyme activity was determined using 1 mM ABTS as substrate. The thermal stability of the purified laccase was determined by following the oxidation of 1 mM ABTS for 2 minutes at 30 ^{o}C after pre-incubation in 20 mM potassium phosphate buffer, pH 7.0, containing 1 µg ml^{-1} BSA for 60 mins at a temperature range of 30 to 90 ^{o}C.

5.2.7 Effects of chloride ion, metal ions and inhibitors

The effects of chloride ion, metal ions, and inhibitors on laccase activity were determined using 1 mM ABTS as a substrate in 100 mM sodium tartrate buffer (pH 2.0) in the presence of chloride, metal ions, and inhibitors at appropriate concentrations.

5.2.8 N-terminal amino acid sequence

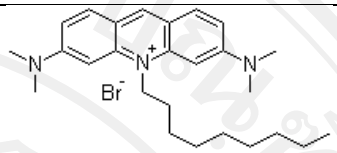
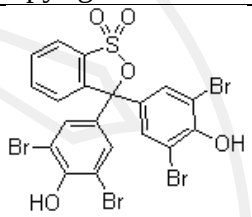
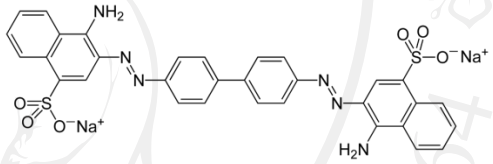
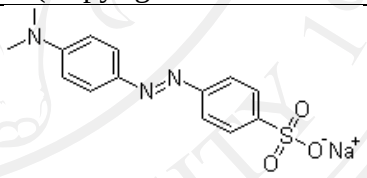
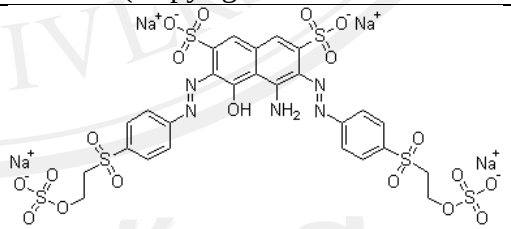
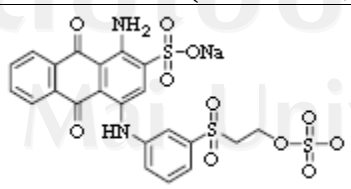
Purified laccase (2 µg) was loaded onto a 10 % SDS-polyacrylamine gel and electroblotted to a polyvinylidiene difluoride membrane (Immobilon-P, Millipore). The amino acid sequence was analyzed by a precise protein sequencing system (Applied Biosystems, Foster City, CA).

5.2.9 Decolorization of synthetic dyes

The decolorization of synthetic dyes was investigated with six chemically different dyes: triphenlymethane dye (Bromophenol Blue; BRB), antraquinone dye (Remazol Brilliant Blue R; RBBR), azo dye (Methyl Orange; MO), diazo dyes (Relative Black 5; RB5 and Congo Red; CR), and heterocyclic dye (Acridine Orange; AO). The characteristics of each dyes were summarized in Table 5.1. The reaction mixture containing 0.01 % of each synthetic dye and 0.45 U/ml of purified laccase in 100 mM sodium acetate buffer (pH 4.0) in the presence or absence of redox mediator, 1-hydroxybenzotriazole; HBT (2 mM). All the reactions were incubated at 28^{o}C without shaking and in complete darkness for 24 hr. Decolorization was determined spectrophotometrically by measuring the decrease in the absorbance at maximum wavelength for each dye. Decolorization was evaluated as follow:

Decolorization(%)= [(initial absorbance)–(final absorbance)]/(initial absorbance)×100

Table 5.1 Characteristics of synthetic dyes

Dyes	λmax (nm)	MW	Structure
Acridine Orange	490	327	 (Copyright 2013 © chemBlink)
Bromophenol Blue	590	670	 (Copyright 2013 © chemBlink)
Congo Red	532	697	 (Copyright 2013 © chemBlink)
Methyl Orange	464	327	 (Copyright 2013 © chemBlink)
Relative Black 5	597	992	 (Claus *et al*., 2002)
Remazol Brilliant Blue R	595	627	 (Osma *et al*., 2007)

5.2.10 Bisphenol A degradation

Bisphenol A (0.01 %) was incubated in reaction mixture containing 0.64 U/ml of purified laccase in 100 mM sodium acetate buffer (pH 4.0) at 28°C and dark condition. The reaction mixture in the presence or absence of redox meditor, 2 mM HBT were incubated for 12 hr and the remaining amount of bisphenol A was quantified at intervals by reverse-phase HPLC.

5.2.11 Quantitative analysis of bisphenol A by High Performance Liquid Chromatography (HPLC)

Bisphenol A was quantitatively analyzed using a Develosil ODS-5 column (4.6 × 250 mm; Nonura Chemicals, Seto, Japan). The fractions were eluted by using a linear gradient of water-methanol at a flow rate of 0.8 ml/min. The gradient program was 0-5 min (methanol 0 %), 5-35 min (methanol 0-100 %) and 35-40 min (methanol 100 %). The eluted sample was monitored by UV absorbance at 230 nm. The peak area on the chromatogram was used to calculate the remaining amount of bisphenol A as a percentage of the initial value.

5.2.12 Analysis of biphenol A degrading products by Gas Chromatography-Mass Spectrometry (GC-MS)

The oxidative degradation products of bisphenol A were identified by using GC-MS. A reaction mixture containing 0.01 % bisphenol A and 1.5 U/ml of laccase from *T. polyzona* in 100 mM sodium acetate buffer (pH 4.0) was incubated with or without HBT at 28°C for 24 hr. To identify the reaction product in the aqueous solution, the reaction mixture was centrifuged at 14,000 g for 15 min to remove the water-insoluble precipitate and then the products in supernatant were extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness, and the resulting

residue was redissolved in 100 µl of ethyl acetate. Five µl of the solution was injected into the HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) which was interfaced with a JMS-SX102A mass spectrometer (JEOL, Tokyo, Japan). Gas chromatographic separation was performed in an Inertcap 1 MS column (30 m × 0.25 mm ID; GL Sciences, Tokyo, Japan). The oven temperature was programmed to rise from 50^{o}C to 300^{o}C at 10^{o}C per min, and MS was taken at 70eV.

5.3 Results and discussion

5.3.1 Purification of laccase

A summary of the purification procedure was shown in Table 5.2. Five purification steps were required to separate laccase from other proteins, polysaccharides and pigments. The purification of laccase started from 740 ml of the culture extract with 557 U of total activity, 409 mg of total protein, and specific activity of 1.36 U/mg protein. Approximately 45-fold purification was achieved with a yield of 10.2 %. Based on native-PAGE with activity staining using 2,6-DMP substrate, it was found that *T. polyzona* secreted one isozyme under SSF conditions, (Fig. 5.1a). The purified laccase showed a single protein band on SDS-PAGE. The molecular mass was estimated to be about 71 kDa by SDS-PAGE (Fig. 5.1b) and 68 kDa by gel filtration, indicating that laccase of *T. polyzona* has a monomeric structure. This result is consistent with most laccases which are monomeric glycoproteins having a molecular mass of 50 - 80 kDa (Mario *et al*., 2002; Rosana *et al*., 2007; Nitheranont *et al*., 2011).

5.3.2 Absorption spectrum

The absorption spectrum of the enzyme shows a shoulder at around 300 nm, corresponding to a type-3 binuclear copper and a peak at around 600 nm corresponding to a type-1 or blue copper atom (Fig. 5.2), which is responsible for the blue color of the concentrated enzyme. Many fungal studies showed blue copper laccase (Maria *et al*., 2000; Mario *et al*., 2002; Nitheranont *et al*., 2011).

Table 5.2 Purification of laccase from *Trametes polyzona* WR710-1

Purification step	total activity (U)	total protein (mg)	Specific activity (U/mg)	purification (fold)	yield (%)
Culture extract	557	409	1.36	-	100
Toyopeal Butyl-650 M	332	30.5	10.9	8.01	59.6
Hiprep 16/10 Q XL	181	12.3	14.8	10.9	32.6
Hiload 16/10 Phynyl Sepharose HP	147	6.47	22.8	16.8	26.5
Hiprep 16/10 Q XL	95.3	2.69	35.5	26.0	17.1
Hiload 16/60 Superdex 200 pg	57.1	0.940	60.8	44.7	10.2

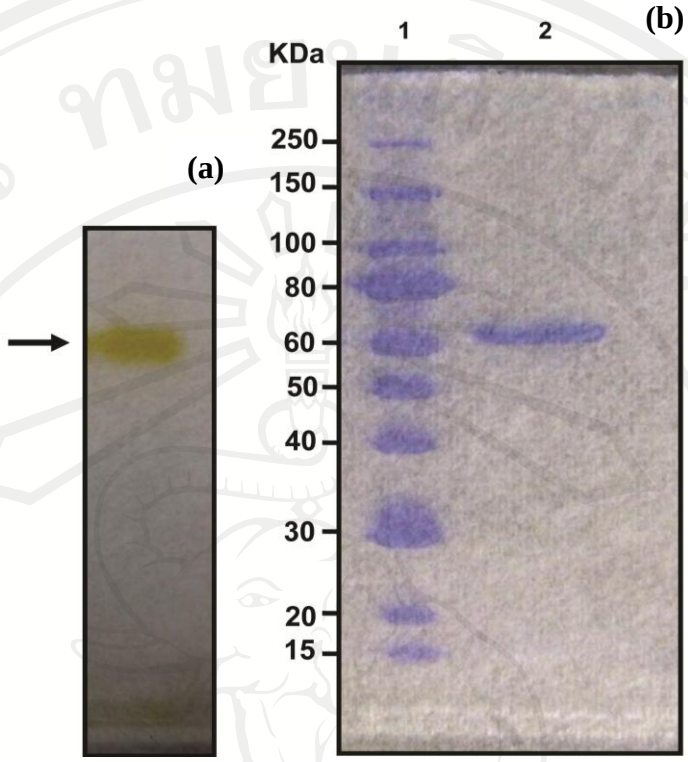


Fig. 5.1 PAGE analysis of laccase.

(**a**) Laccase activity staining with 2,6-DMP as substrate for native-PAGE of culture extract of *T. polyzona*. The arrow shows protein band having laccase activity. (**b**) SDS-PAGE of the purified laccase. Lane 1, Molecular weight marker, Lane 2, Purified laccase (0.1 µg).

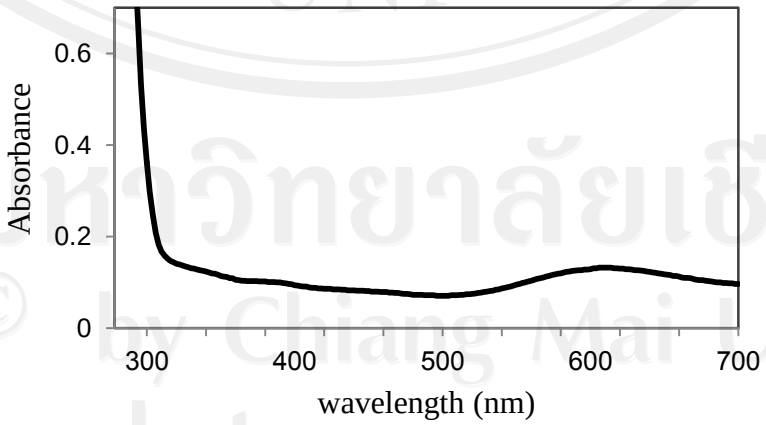


Fig. 5.2 Absorption spectrum of purified laccase from *T. polyzona* WR710-1.

5.3.3 Effects of pH and temperature on laccase activity and stability

The optimal pH values for laccase production from *T. polyzona* was studied. It was found that the optimum pH were 2.0 for ABTS, 4.0 for L-DOPA, guaiacol and catechol, and 5.0 for 2,6-DMP substrates (Fig. 5.3a).This result is identical to the optimum pH observed for *Coriolus hirsutus* laccase (Lee and Shin, 1999)**.** Many fungal laccases have been reported to have such a variation in the optimum pH for different substrates (Christiane *et al*., 2002; Nagai *et al*., 2002; Jia and Yi, 2004). The effect of pH on enzyme stability was determined (Fig. 5.3b). It was found that the *Trametes* laccase had a high stability between pH 6.0 to 8.0 using ABTS as substrate, which is similar to the previous study by Harnándes Fernaud *et al*., 2006. Moreover, above which the enzyme was rapidly inactivated, only about 1 % of the initial laccase activity was retained at pH 10.0.

The thermal stability of the purified laccase was determined by following the oxidation of 1 mM ABTS. As shown in Fig. 5.3c, the purified laccase was stable up to 40 ^{o}C, with no activity loss. Ninety percent of the laccase activity was retained when kept at 50 ^{o}C and no activity was retained above 70 ^{o}C. The laccase of *Pleurotus ostreatus* also showed a similar pattern in its thermal stability curve (Okamoto *et al*., 2000).

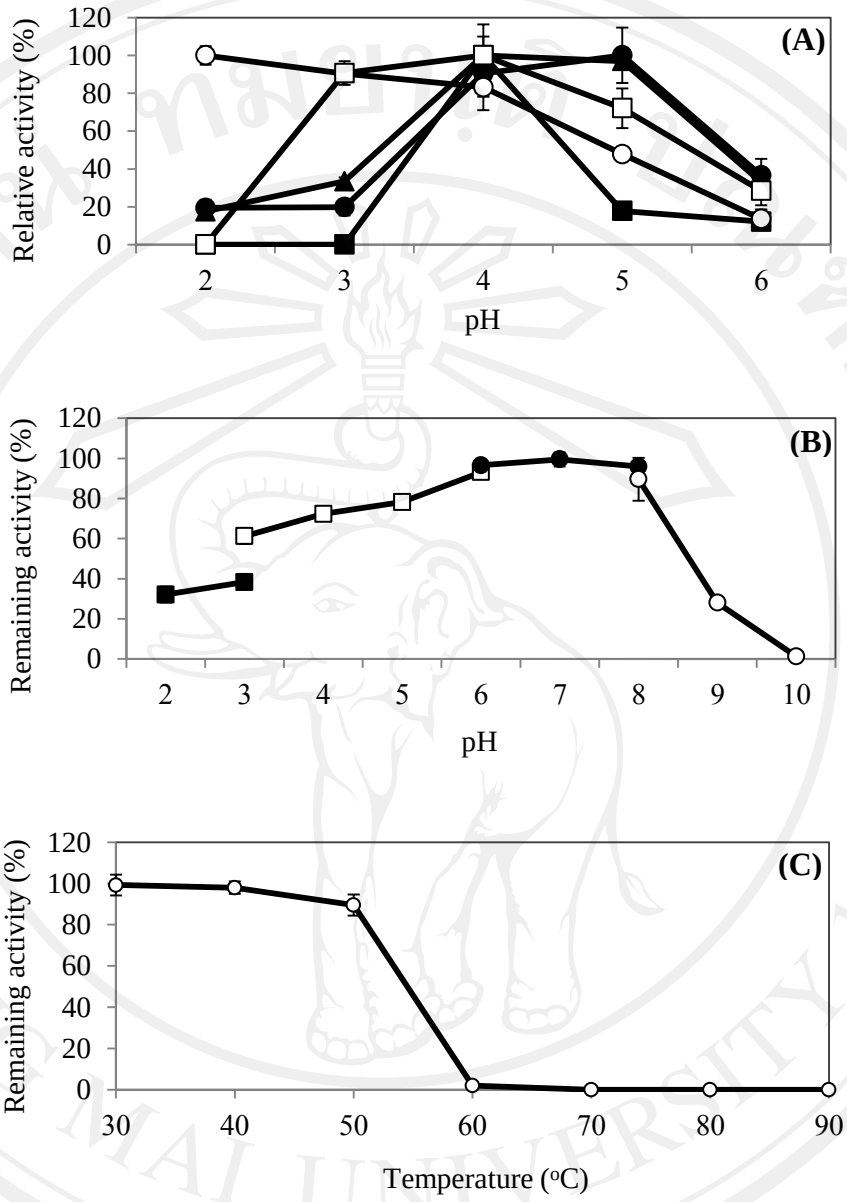


Fig. 5.3 Effects of pH and temperature on laccase activity and stability.

(A) Effect of pH on laccase activity with ABTS (○), 2,6-DMP (●), guaiacol (▲), catechol (□), and L-DOPA (■) as substrate. (B) Effect of pH on laccase stability. Remaining activities were determined after incubation of laccase for 24 h in sodium tartrate buffer pH 2.0-3.0 (■), sodium acetate buffer pH 3.0-6.0 (□), potassium phosphate buffer pH 6.0-8.0 (●), and Tris-SO_4 buffer pH 8.0-10.0 (○). (C) Effect of temperature on the stability of laccase. Remaining activities were determined after incubation of laccase for 60 min at various temperatures.

5.3.4 Substrate specificity and kinetic properties of laccase

The substrate specificity of the laccase was evaluated using different phenolic compounds (2,6-DMP, guaiacol, catechol, and L-DOPA) and a non phenolic heterocyclic compound (ABTS) at the optimum pH for each substrate. Laccase oxidized phenolic and non phenolic compounds with considerably different kinetic constants (Table 5.3). Among the substrates tested in this study, the highest catalytic efficiencies k_{cat}/K_m and the highest affinity (the lowest of K_m value) were found for ABTS substrate. Laccase from *T. polyzona* did not oxidize L-tyrosine and veratryl alcohol, which are standard substrates for tyrosinase and arylacohol oxidase, respectively. The highest kinetic properties of *T. polyzona* laccase were found for ABTS substrate which is similar to other fungal laccase reports (Nitheranont *et al*., 2011; Murugesan *et al*., 2006). The *Trametes* laccase was also found to oxidize typical laccase phenolic substrates. These catalytic properties are consistent with those of laccases from many basidiomycetes such as *T. pubescens* (Christiane *et al*., 2002), *T. trogii* (Héla *et al*., 2006), *Grifola frondosa* (Nitheranont *et al*., 2011) and *Pycnoporus coccineus* (Atef *et al*., 2005).

Table 5.3 Substrate specificity and kinetic constants of laccase from *Trametes polyzona* WR710-1

substate	optimum pH	λ measured (nm)	ε (cm^{-1} mM^{-1})	*V*max (mM min^{-1})	*K*m (mM)	k_{cat} (s^{-1})	k_{cat}/*K*m (s^{-1} mM^{-1})
2,6-DMP	5.0	470	49.6	0.235	0.503	38.2	75.9
L-DOPA	4.0	460	3.9	0.514	6.28	83.2	13.3
Guaiacol	4.0	470	12.0	0.570	1.89	92.2	48.7
Catechol	4.0	450	2.2	0.772	4.08	125	30.6
ABTS	2.0	420	36.0	1.84	0.150	594	3960

5.3.5 Inhibitory effect of chloride ion on laccase activity

The activity of purified laccase was inhibited by increasing chloride ion concentrations (Fig. 5.4). However, almost full activity was recovered after removal of NaCl by dialysis indicating that the inhibitory effect of chloride ion is reversible. To evaluate the common inhibition by chloride ion of fungal laccases, we studied the effect of chloride on the activity of commercial *T. versicolor* laccase (Jena Bioscience, Jena, Germany). As shown in Fig. 5.4, the chloride ion also inhibited the activity of *T. versicolor* laccase. This result is consistent with previous studies on laccase from different fungi, including those from *Trichophyton rubrun* (Jung *et al*., 2002), *Marasmius quercophilus* (Farnet *et al*., 2008) and *G. frondosa* (Nitheranont *et al*., 2011). An inhibition mechanism of laccase by the chloride ion has been suggested by Naki and Varfolomeev (1981) as follows: chloride ion acts as a competitive inhibitor with the electron donor and blocks the electron pathway at the active site of the laccase. Moreover, the degree of inhibition by chloride or other halide ions seems to be linked to the availability of copper atoms in the active site (Abadulla *et al*., 2000).

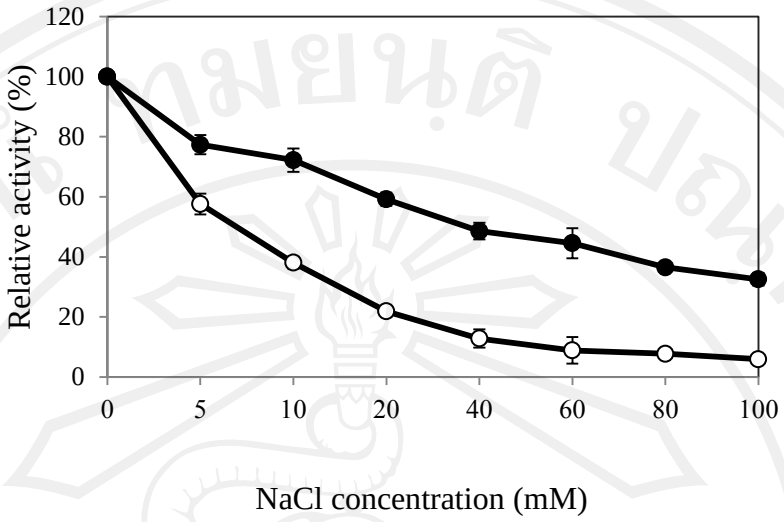


Fig. 5.4 Inhibitory effect of chloride ion on the laccase avtivities.
The activities of laccase from *T. polyzona* (●) and the laccase from *T. versicolor* (○) were determined in the presence of NaCl at various concentrations.

5.3.6 Effect of metal ions and inhibitors on laccase activity

The inhibition of laccase activity by metal ions has been reported for some other fungal laccases including those of *Lentinula edodes* (Nagai *et al*., 2002) and *M. quercophilus* (Farnet *et al*., 2008). Similarly, metal ions exhibited inhibitory effects on the activity of *T. polyzona* laccase. As shown in Table 5.4, the monovalent ions, Na^{+} and K^{+}, were less inhibitory on laccase activity than the metal ions, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Al^{3+} and Cr^{3+} which exhibited inhibitory effect at 5 mM concentrations of 3, 6, 19, 28, 19, 14 and 23 %, respectively. Among the metal ions tested, Fe^{2+} was the most efficient inhibitor of laccase causing more than 95 % inhibition at 5 mM. The laccase of *T. polyzona* was also inhibited by Cu^{2+}, although laccase is a copper-containing enzyme. Okamoto *et al*. (2000) suggested that an excess supply of Cu^{2+} ions might cause a change in the structure of laccase leading to a loss of activity.

Table 5.4 Effects of metal ions on purified laccase from *Trametes polyzona* WR710-1

Metal salt	Concentration (mM)	Relative activity (%)
None	-	100
Na_2SO_4	5	93
K_2SO_4	5	86
MgSO4	5	97
$FeSO_4.7H_2O$	1	6.8
	5	4.2
$ZnSO_4.7H_2O$	1	94
	5	81
	10	74
$CoSO_4$	1	87
	5	72
	10	64
$CuSO_4$	1	87
	5	81
$K_2Al_2(SO_4)_4.24H_2O$	5	86
	10	83
$CrK(SO_4)_2.12H_2O$	1	88
	5	77

The sensitivity of laccase towards various inhibitors was also tested and the result is shown in Table 5.5. Laccase from *T. polyzona* was completely inhibited by 0.1 mM NaN_3, 1 mM dithiotheitol and 10 mM L-cysteine. The metal chelator EDTA

had only a slight inhibitory effect (10 % inhibition at 20 mM). The results are consistent with the report by Rosana *et al*. (2007) who showed that laccase from *T. versicolor* was completely inhibited by 0.001 mM NaN_3, 0.5 mM dithiothreitol and 0.5 mM L-Cysteine. A strong inhibitory effect of azide on laccase activity has been explained as follows: azide binds to the copper atoms in the protein structure and blocks electron transfer leading to a loss of catalytic activity (Sugumaran, 1995). In addition, the inhibition by thiol compounds, dithiothreitol and L-cysteine was presumed to be the result of coordination of the thiol to the copper atoms in the enzyme active site (Wells *et al*., 2006).

Table 5.5 Effects of Inhibitors on purified laccase from *Trametes polyzona* WR710-1

Inhibitors	Concentration (mM)	Inhibition (%)
none	-	0
EDTA	1	0.70
	10	5.2
	20	9.9
NaN_3	0.01	56
	0.1	100
L-Crsteine	1	60
	5	76
	10	100
Dithiotheitol	1	100
	5	100
	10	100

5.3.7 N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified laccase was determined to be AVTPVADLQISNAGISPDTF. These 20 residues were compared with those reported for laccases from other fungi (Table 5.6). This is the first report on the N-terminal amino acid sequence of the *T. polyzona* laccase which was highly similar to those of other white-rot basidiomycetes laccases, including those of *Coriolopsis gallica* (Dong *et al*., NCBI/GenBank database Accession No. AAW65485.1), *Rigidoporus microporus* (Liu *et al*., 2003) and *Polyporus brumalis* (Ryu *et al*., 2008) with 75 % identity. It was also found to have more than 60 % identity with those of other white-rot basidiomycetous fungal laccases like those of *Trametes sp. C30* (Cusano *et al*., 2009), *T. pubescens* (Galhaup *et al*., 2002), *P. coccineus* (Hoshida *et al*., 2001) *and T. versicolor* (Jolivalt *et al*., 2005). In contrast, the N-terminal sequences of laccases from non-wood-rotting fungi, the ascomycetes, *Neurospora crassa* (Germann *et al*., 1988) and *Melanocarpus albomyces* (Kiiskinen and Saloheimo, 2004) were significantly different from that of *T. polyzona* laccase (only 10 and 5 % identities, respectively).

Table 5.6 Comparison of N-terminal amino acid sequence of laccase from *Trametes polyzona* WR710-1 to those of other fungal laccases

Fungi	N-terminal amino acid sequence																				references
Trametes polyzona	A	V	T	P	V	A	D	L	Q	I	S	N	A	G	I	S	P	D	T	F	this study
Coriolopsis gallica	A	I	G	P	V	A	D	L	T	I	S	N	A	N	I	S	P	D	G	F	GenBank No. AAW65485.1
Rigidoporus microporus	A	I	G	P	V	A	D	L	H	I	S	N	A	N	I	S	P	D	G	F	Liu *et al*., 2003
Polyporus brumalis	A	I	G	P	V	A	D	L	T	I	S	N	A	D	I	S	P	D	G	F	Ryu *et al*., 2008
Trametes sp. C30	A	L	G	P	V	A	D	L	V	I	S	N	A	A	I	A	P	D	G	F	Cusano *et al*., 2009
T. pubescens	G	I	G	P	V	A	D	L	T	I	S	N	A	A	V	S	P	D	G	F	Galhaup *et al*., 2002
Pycnoporus coccineus	A	I	G	P	V	A	D	L	T	L	T	N	A	A	V	S	P	D	G	F	Hoshida *et al*., 2001
T. versicolor	G	I	G	P	V	A	D	L	T	I	T	N	A	A	V	S	P	D	G	F	Jolivalt *et al*., 2005
Neurospora crassa	G	G	G	G	G	C	N	S	P	T	N	R	Q	C	W	S	P	G	F	N	Germann *et al*., 1988
Melanocarpus albomyces	A	P	P	S	T	P	A	Q	R	D	L	V	E	L	R	E	A	R	Q	E	Kiiskinen and Saloheimo, 2004

The identity of N-terminal amino acid sequence inferred from the results of Blast search in NCBI database. Identical corresponding amino acid residues are highlight.

5.3.8 Synthetic dyes decolorization

Different synthetic dyes were decolorized by purified laccase from *T. polyzona* WR710-1. As shown in Fig. 5.5, More than 80% of BPB, RBBR and MO were decolorized even in the absence of HBT. Moreover, 72, 52 and 27% decolorization were achieved with CR, RB5 and AO, respectively. The addition of 2 mM HBT as a redox mediator into the reaction mixtures led more decolorization percentages, around 36 and 24% for RB5 and AO which were significantly increased values when compared to non HBT condition.

Laccase may be involved in the oxidation of the phenolic group of azo dye to produce a radical at the carbon bearing the azo linkage (Chivukula and Renganathan, 1995). Each dye was not degraded in the same extent. This can be explained in terms of the structure and size of each dye, a low number of aromatic rings and the simple molecule is degraded more rapidly than the complex molecule (Cameselle *et al*., 2003). Claus (2002) found that the system of laccase plus mediator enhanced the dye decolorization rate and decolorized some dyes resistant to laccase alone. The effect of redox mediator on synthetic dye decolorization by laccase was extensively discussed in previous studies (Rodríguez *et al*., 2005; Mariam *et al*., 2006 and Rim *et al*., 2010).

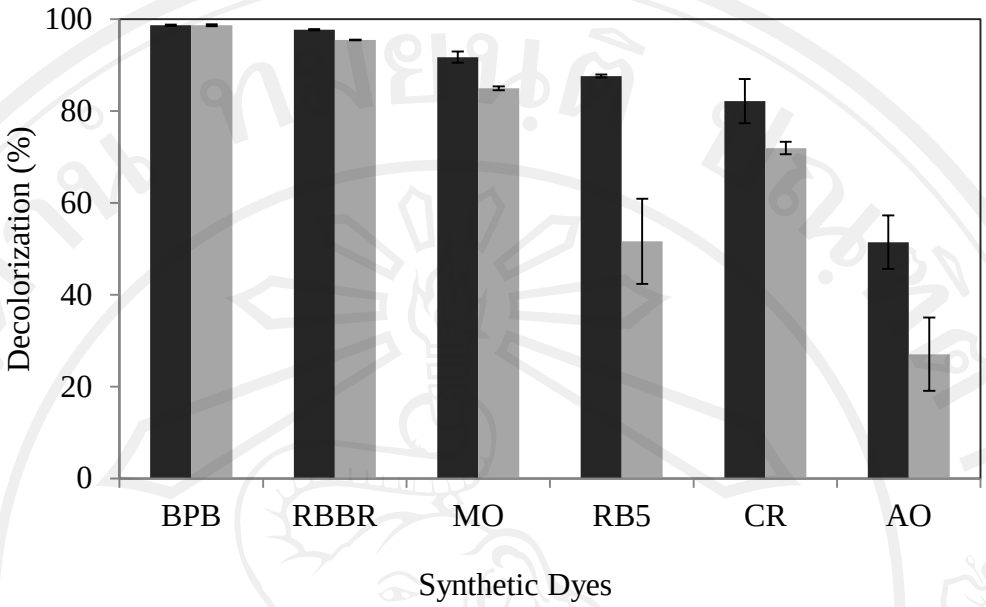


Fig. 5.5 Decolorization of syethetic dyes by purified laccase from *T. polyzona* WR710-1. Percentages of decolorization in the presence (black bars) or absence (gray bars) of HBT after 24 h of incubation.

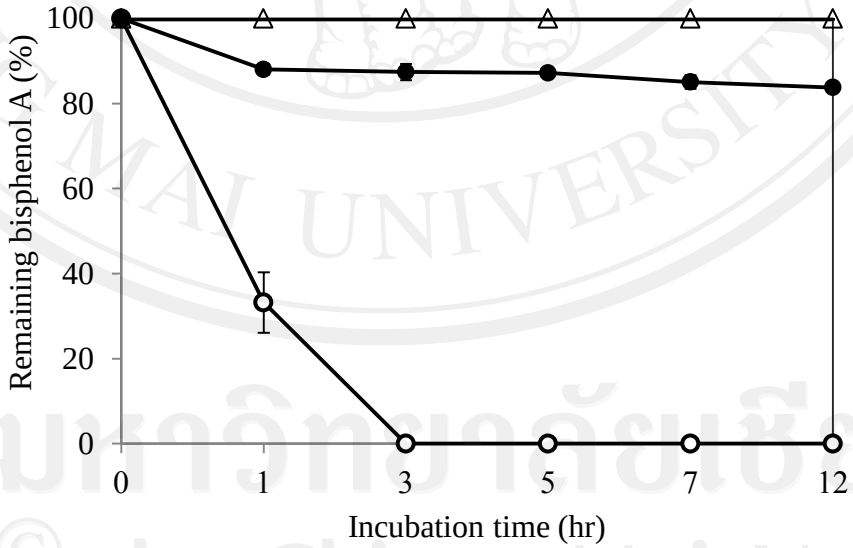


Fig. 5.6 Time-course of bisphenol A oxidation catalyzed by the purified laccase from *T. polyzona* WR710-1. Control reaction without laccase (△); absence of HBT (●); presence of HBT (○).

5.3.9 Bisphenol A degradation

It was shown that bisphenol A was biodegraded by purified laccase from *T. polyzona*. As shown in Fig. 5.6, 12% of initial bisphenol A was removed within 1 hour and there after the degradation rate was declined in the absence of HBT. The oxidation of bisphenol A can be improved remarkably by the addition of HBT. Bisphenol A was 66.8% removed within 1 hr and completely removed within 3 hr. Thus, bisphenol A oxidization by laccase was much more effective in the presence of HBT than in laccase alone. Laccases are able to degrade bisphenol A toxin, in agreement with several reports (Fukuda *et al*., 2001; Uchida *et al*., 2001 and Saito *et al*., 2004).

5.3.10 Isolation and identification of reaction products of bisphenol A degradation

In order to identify the reaction product from bisphenol A degradation in the aqueous solution (the reaction in the absence of HBT), GC-MS analysis were conducted (Fig. 5.7). The GC chromatogram demonstrated two major peaks (A and B) (Fig. 5.7a). Peak A were determined to be 4-isopropenylphenol exhibiting m/z (relative intensity, % in parentheses) of 134(M^+, 100), 119 (76), 91(29), 77(11), and 65(9), whereas Peak B was determined to be the remaining of bisphenol A exhibiting m/z of 228(M^+, 21), 213(100), 119(19), 107(7), 91(10), 77(4) and 65(5). These data show that the major product from the biodegradation of bisphenol A by laccase from *T. polyzona* was 4-isopropenylphenol. Our results are in agreement with several reports with fungal laccase, *Trametes villosa* (Uchida *et al*., 2001), *Trametes* sp. (Michizoe *et al*., 2005) and *Grifola frondosa* (Nitheranont *et al*., 2011). A portion of bisphenol A in the reaction mixture may be polymerized by laccase to form water-

insoluble, high molecular weight compounds, such as oligomers (Uchida *et al*., 2001). The addition of HBT in reaction mixture remarkably improved the oxidation rate of bisphenol A, as the precipitate was found in reaction mixture. GC-MS analysis of this reaction in the presence of HBT showed that the peak of 4-isopropenylphenol was detected only in the early period of the reaction and completely disappeared after 3 hr, indicating that 4-isopropenylphenol which was generated in the oxidative degradation of bisphenol A, was completely polymerized to give a water insoluble product by the action of the HBT radical formed by laccase. Indeed the relatively intensive peak benzotriazole, the reduction product of HBT radical (Li *et al*., 1998; Potthast *et al*., 2001) was detected the GC-MS profile (Fig. 5.7b, peak C).

Unless laccase was used in pretreatment process for bio-ethanol production, it was showed that laccase was used for detoxification. In this study, this is the first report on a potential of laccase from *T. polyzona* as an effective enzyme for environmental applications especially decolorization of synthetic dyes and bisphenol A degradation.

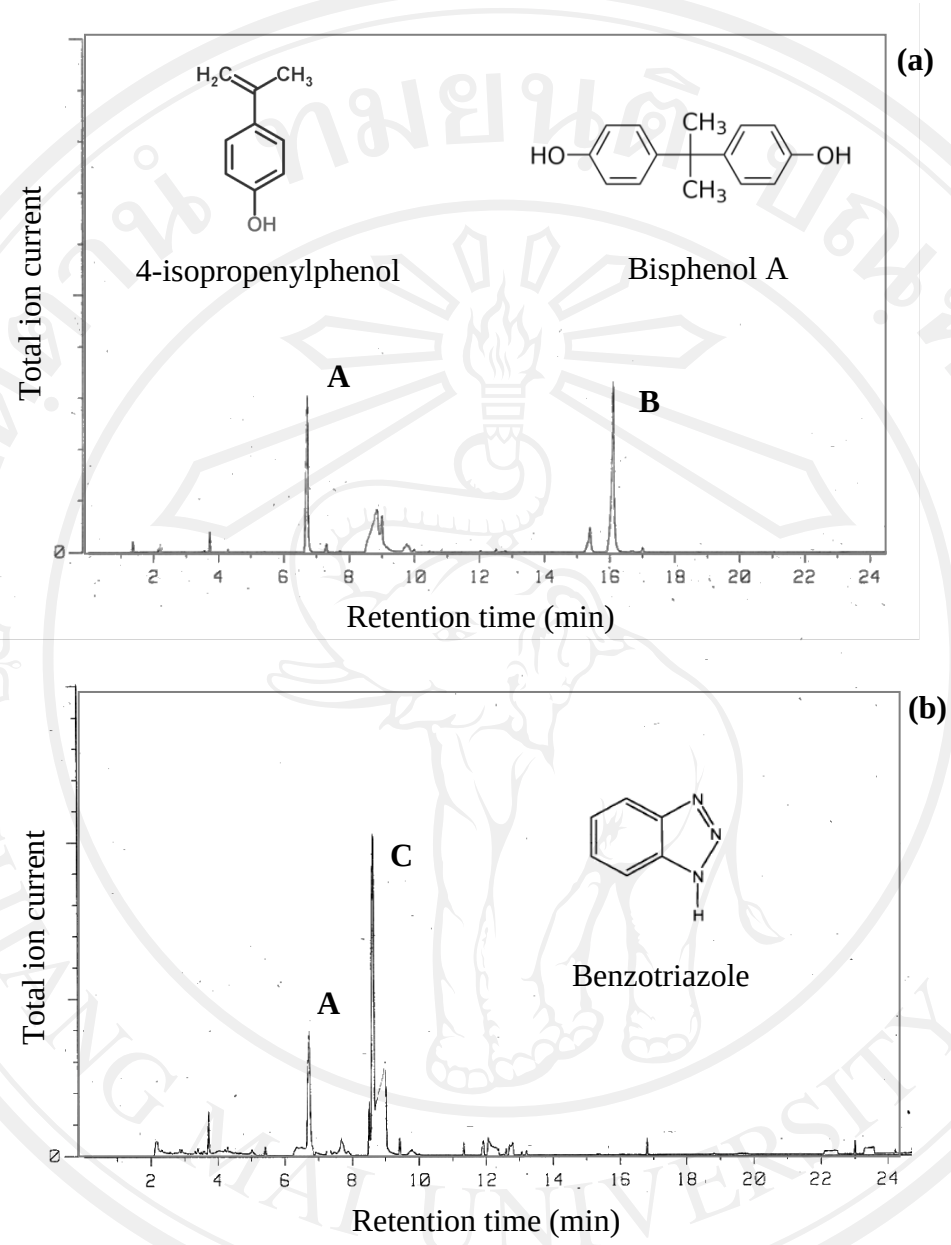


Fig. 5.7 GC-MS analysis of the reaction product from the bisphenol A degradation by laccase of *T. polyzona* WR710-1.

(**a**): The reaction in the absence of HBT; (**b**): The reaction in the presence of HBT. The mass spectral data are expressed by m/z (relative intensity, %); Peak A: 134(M^+, 100), 119(76), 91(29), 77(11), and 65(9), Peak B: 228(M^+, 21), 213(100), 119(19), 107(7), 91(10), 77(4) and 65(5) and Peak C: 119(M^+, 100), 91(88), 63(30), and 52(11).