Enzymatic modification of 2,6-dimethoxyphenol for the synthesis of dimers with high antioxidant capacity

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Abstract

2,6-Dimethoxyphenol is a phenolic compound that is extensively used for the measurement of laccase activity, but is often not exploited for its potential as an antioxidant compound. Since laccase can be used to modify phenolic antioxidants as a way of improving their activities, the present study investigated the laccase-mediated oxidation of 2,6-dimethoxyphenol in biphasic or homogenous aqueous-organic media for the production of compounds with higher antioxidant capacity than the starting substrate. The main product was a dimer (*m/z* 305.0672), which was further characterized as a symmetrical C-C linked 3,3',5,5'-tetramethoxy biphenyl-4,4'-diol. In the monophasic system, the dimer was preferentially formed when acetone was used as co-solvent, while in the biphasic system, formation of the dimer increased as the concentration of ethyl acetate was increased from 50 to 90 %. The dimer showed higher antioxidant capacity than the substrate (≈ 2×) as demonstrated by standard antioxidant assays (DPPH and FRAP). These results demonstrate that a product of the laccase-catalysed oxidation of 2,6-dimethoxyphenol can find useful application as a bioactive compound.

Key words: 2,6-dimethoxyphenol, antioxidant capacity, laccase

1. Introduction

Increased interest in the use of natural phenolic compounds as antioxidants and their potential related health benefits [1,2] have become apparent in recent times. These are part of a number of secondary metabolites found in plants that aid in the protection of plant tissue against insects, infections, pathogen attack and UV radiation [3,4]. Generally, antioxidants are an important class of compounds, that when present at low concentrations relative to an oxidizable substrate, significantly delay, retard or inhibit oxidation of that substrate. Phenolic antioxidants, in particular, are known to act as terminators of free radicals [5]. A major feature of free radicals is that they have extremely high chemical reactivity (due to the presence of unpaired electrons), which accounts for the reasons why they inflict damage in cells [6]. Since cell damage caused by free radicals has been implicated in various pathophysiological conditions such as liver cirrhosis, atherosclerosis, cancer, diabetes, neurological disorders, ischemia/reperfusion, and aging [7-11], compounds that can scavenge free radicals have great potential in ameliorating these disease processes [12-15]. Attention is being increasingly shifted away from synthetic antioxidants due to potential health hazards and thus plant phenols are increasingly becoming a subject of intensive research due to their bioactive properties and much reduced adverse side effects.

2,6-Dimethoxyphenol (2,6-DMP) and its derivatives, are plant phenols that form the predominant smoke component of thermal degradation products from hardwood. It forms about 70-80 % of total methoxyphenols in birchwood smoke which are of great importance for the smoke flavour, preservation and antioxidant effect [16]. As a major component of birchwood smoke, its antioxidant capacity is stronger than the 2-methoxyphenols that are present in lower amounts [17]. 2,6-DMP is widely documented as a substrate in the determination of laccase

activity. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper-containing enzymes which reduce molecular oxygen to water and simultaneously perform one-electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines [18]. Laccases have been reported to catalyze the oligomerisation or polymerization of many phenolic compounds as a way of increasing antioxidant capacity [19-21].

Although some products of laccase-catalysed oxidation of 2,6-DMP have been characterised [22-24], the studies mainly focused on determining the suitability of its use in laccase assays [23]. Application of the 2,6-DMP oxidation products as antioxidants in their own right has not yet been investigated. As part of our attempts to enzymatically modify phenolic molecules as a way of enhancing their bioactive properties [19,25-27], this work investigates the potential of the laccase produced by *Trametes pubescens* to modify 2,6-DMP as a way of increasing its antioxidant capacity

2. Materials and methods

2.1. Chemicals and enzyme

2,6-DMP and other chemicals were purchased from Sigma–Aldrich, South Africa. Extracellular laccase was produced by fermentation of the white rot fungal strain *Trametes pubescens* (strain CBS 696.94) using an airlift reactor, and purified as described by Ryan et al. [28]. The *T. pubescens* strain was obtained from the Institute of Applied Microbiology, University of Natural Resources and Life Sciences (Vienna, Austria) and is currently deposited in the stock culture collection at the Biocatalysis and Technical Biology Research Group, Cape Peninsula University

of Technology. The culture is maintained on 3 % (w/v) malt extract agar plates at 4 ^{o}C and sub-cultured every 60 days to maintain viability.

2.2. Enzyme activity

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2,'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, ε_{420} = 36,000 $M^{-1}cm^{-1}$) as the substrate [29]. The reaction mixture contained 0.330 ml ABTS (5 mM), 2.5 ml 0.1 M sodium acetate buffer pH 5.0 (previously determined as the optimum pH for *T. pubescens* laccase) and 0.17 ml laccase. Oxidation of the ABTS was monitored by measuring the increase in absorbance at 420 nm. One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute at 25 °C.

2.3. Oxidation of 2,6-DMP

A biphasic system comprising buffer with ethyl acetate as co-solvent or a monophasic system with miscible solvents (methanol, ethanol, toluene or acetone) as co-solvents was employed for the oxidation reactions. For the biphasic system the reaction mixture contained 2,6-DMP (10 mM), laccase (10 U) in 100 mM sodium acetate buffer (pH 5.0) and ethyl acetate. The effect of ethyl acetate on product formation was tested by varying its concentration (50, 60, 70, 80, 90, and 95%, v/v) and the effect of reaction time on product formation was also determined (1 – 8 hours; time variation only performed using 90 %, v/v, ethyl acetate – previously determined as the optimum co-solvent concentration). In the monophasic system the miscible solvents were used at 80 %, v/v (previously determined as the optimum for product yield and reduced side reactions). The reactions were carried out for 24 hours at 28 °C with shaking, using

an orbital shaker at 180 rpm. The reactions were monitored by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

2.4. Thin Layer Chromatography (TLC)

TLC analysis was performed on aluminium–backed silica gel 60 F254 (Merck) plates using heptane: ethyl acetate: acetic acid (1:1:0.05, v/v/v) as the mobile phase. The compounds were visualized by exposure to UV light at 254 nm.

2.5. High Performance Liquid Chromatography (HPLC)

When miscible solvents were used, an equal volume of ice cold methanol (99.8 %) was added to the reaction mixtures to precipitate out the protein. The mixture was allowed to stand on ice for 20 min before centrifugation at 0 ºC for 15 min at 14,000 x g. In the biphasic system, however, the enzyme was readily separated from the product. The supernatant (1.2 ml aliquots) was transferred into clean vials and analysed by HPLC. HPLC analysis was carried out using a Dionex UltiMate 3000 HPLC system (Dionex Softron, Germany) equipped with a 3000RS pump, WPS 3000RS autosampler and a DAD-3000RS detector. Separation of the reaction products was carried out on a reversed phase Sunfire C18 column (5µm, 4.6×150 mm) (Waters, Ireland) using a linear gradient of 0.1 % v/v formic acid (solvent A) and acetonitrile (99.9 %) (solvent B) at a flow rate of 0.5 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30 ^{o}C. The gradient set up was as follows: 98 % A to 0 % A (20 min); 0 % A to 98 % A (20-21 min); 98 % A (21-23 min). The products were monitored and detected at 280 nm.

2.6. Product purification

The reaction products were purified by flash chromatography. The miscible solvents containing products were evaporated using a rotary evaporator (at 60 ºC) (Heidolph, Germany) and the product extracted with ethyl acetate followed by separation using a separation funnel. The aqueous phase was washed twice with ethyl acetate and monitored for the absence of product. The organic phase was dried using a rotary evaporator (Heidolph, Germany). For the biphasic system, the organic phase was separated using a separation funnel and the aqueous phase washed twice with ethyl acetate. The organic phase was evaporated (at 60 ºC) under reduced pressure with a rotary evaporator and the crude residue purified by silica flash chromatography using heptane: ethyl acetate: acetic acid (1:1:0.05, v/v/v) as mobile phase. The pure fractions were dried using a rotary evaporator and the products sequentially washed with acetone, methanol and then acetone again to remove the acetic acid.

2.7 Product characterisation

The purified product was characterised by mass spectrometry and Nuclear Magnetic Resonance (NMR) analysis.

2.7.1. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed on a Dionex HPLC system (Dionex Softron, Germering, Germany) equipped with a binary solvent manager and autosampler coupled to a Brucker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). The products were separated by reversed phase chromatography using gradient elution as described above. MS spectra were

acquired in negative mode using the full scan and auto MS/MS (collision energy 25 eV) scan modes with dual spray for reference mass solution. Electrospray voltage was set to +3500 V. Dry gas flow was set to 8 l min^{-1} with a temperature of 220 °C and nebulizer gas pressure was set to 17.5 psi. Smart Formula 3D (combining exact mass and isotopic pattern information in MS and MS/MS spectra) allowed for generation of formula of relevant compounds.

2.7.2. Nuclear Magnetic Resonance (NMR) analysis

NMR spectra were recorded using a VARIAN 200 spectrometer (^{1}H, 200MHz; ^{13}C, 50MHz). The spectra were determined at ambient temperature in deuterated chloroform ($CDCl_3$) and methanol solutions, with $CHCl_3$ at δ 7.26 for ^{1}H NMR spectra and chloroform (δ 77.00) for ^{13}C-NMR spectra as internal standards. In the NMR spectra, assignments of signals with the same superscripts are interchangeable. Splitting patterns are designated as "s", "d", "t", "q", "m" and "bs". These symbols indicate "singlet", "doublet", "triplet", "quartet", "multiplet" and "broad singlet".

2.8. Antioxidant activity determination

Antioxidant activities of the substrate (2,6-DMP) and oxidation product were determined using three methods: DPPH (2, 2' -diphenyl-1-picrylhydrazyl) scavenging effect, TEAC (Trolox equivalent antioxidant capacity) assay, and FRAP (ferric reducing antioxidant power) analysis.

2.8.1 DPPH (2, 2' -diphenyl-1-picrylhydrazyl) scavenging effect

Antioxidant capacity was determined by measuring DPPH radical-scavenging activity [30]. Briefly, 3.9 ml of DPPH dissolved in methanol (0.025 mg/ml) was added to 0.1 ml sample

(dissolved in methanol) at various concentrations. The mixture was shaken vigorously and incubated at room temperature in the dark for 60 min, and the decrease in absorbance at 517 nm determined using a spectrophotometer (Rayleigh UV–9200, China). The remaining concentration of DPPH in the reaction medium was then calculated from a calibration curve obtained with DPPH at 517 nm.

The percentage of remaining DPPH ($DPPH_R$) was calculated as follows:

$$\% \ DPPH_R = [(DPPH)_T/(DPPH)_{T=0}] \ X \ 100$$

where $(DPPH)_T$ is the concentration of DPPH at the time of 60 min and $(DPPH)_{T=0}$ is the concentration of DPPH at time zero (initial concentration). The percentage of remaining DPPH against the sample/standard concentration was plotted to obtain the amount of antioxidant (mM) necessary to decrease the initial concentration of DPPH by 50 % (EC_{50}).

2.8.2. TEAC (Trolox equivalent antioxidant capacity) assay

The ABTS radical scavenging activity of 2,6-DMP and its product were determined according to the method described by Re et al. [31]. The trolox equivalent antioxidant capacity (TEAC) method is based on the ability of antioxidant molecules to quench $ABTS^{\cdot+}$, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water soluble vitamin E analog. The addition of antioxidants to the preformed radical cation decolourizes the $ABTS^{\cdot+}$ as it is reduced to ABTS. $ABTS^{\cdot+}$ solution was prepared 12-16 h before use by mixing ABTS salt (7 mM) with potassium persulfate (2.45 mM) and then stored in the dark until the assay was performed. The $ABTS^{\cdot+}$ solution was diluted with methanol to give

an absorbance of 0.70 ± 0.002 at 734 nm. Each sample (100 µl) prepared at different concentrations was mixed with 1100 µl $ABTS^{\cdot+}$ solution and the absorbance read after a 30 min incubation at 25°C.

2.8.3. FRAP (ferric reducing antioxidant power) analysis

Total antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain [32,33]. The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. The principle behind this is that, at low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorbance at 593 nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio just before use and heated to 37 ^oC. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) with 16 ml glacial acetic acid and brought to 1 l with distilled water. The TPTZ solution was prepared by making a solution of 10 mM TPTZ in 40 mM HCl. Sample (100 µl) was mixed with 3 ml of working FRAP reagent and absorbance (593 nm) is measured at 0 minute after vortexing. Thereafter, samples were placed at 37^oC in a water bath and absorbance measured after 4 min. Ascorbic acid standards (0.1 mM-1.0 mM)

were processed in the same way and a standard curve (Fig. 1) was prepared by plotting the average FRAP value versus its concentration. The calibration curve revealed a highly positive linear relation (R^2 = 0.9882) between the mean FRAP value and the concentration of the ascorbic acid standards. This curve was therefore used to estimate antioxidant potential (FRAP values) of the test samples.

3. Results and Discussion

3.1. Laccase-catalysed oxidation of 2,6-DMP

HPLC analysis of the laccase-catalysed oxidation of 2,6-DMP showed the formation of five products at t_R 9.0 min (P1), t_R 9.5 min (P2), t_R 10.1 min (P3), t_R 10.8 min (P4), and t_R 11.3 min (P5) (Fig. 2). The product P4 was the main product constituting 36.96 % (as determined by peak areas) of the soluble products separated by HPLC compared to P1 (8.25 %), P2 (13.09 %), P3 (19.79 %) and P5 (21.92 %). LC-MS analysis in negative mode showed that both products P1 and P2 had distinct signals at *m/z* 277.07; P3 and P4 had signals at *m/z* 305.07 while P5 had an ion signal at *m/z* 291.09 (Fig. 3). These $[M-H]^-$ ion signals suggest dimerisation of 2,6-DMP to form the main product P4, demethylation of P4 to form P5, and loss of two methyl groups to form P1 and P2. Minor products were observed (only with the more sensitive MS detector) at m/z 263.0637 and m/z 249.0484 suggesting loss of 3 methyl groups and 4 methyl groups by the dimer, respectively. Demethylatyion of methoxy-substituted phenolic substrates is a well known reaction in the modification of structurally similar lignin molecules [34-36]. The dimer, product P4, (*m/z* 305.0672) showed a simple fragmentation pattern in which there was a successive neutral loss of methyl groups (*m/z* 290.0447, -1 methyl group; *m/z* 275.0208, -2 methyl groups; *m/z* 261.0402, -3 methyl groups, and *m/z* 247.0256, -4 methyl groups) (Fig. 4A). Similar

fragmentation patterns were observed with the minor products P2 and P5 (Fig. 4B and 4C, respectively). Fig. 5 shows the predicted mechanism of formation of mass fragments from the main product, P4.

After flash chromatography, the yield of the purified main product (*m/z* 305.0672) was 20.91 % (expressed as a percentage of the starting material). NMR analysis of the product (Fig. 6) showed that the dimer was linked through a C-C linkage to form a 3,3',5,5'-tetramethoxy biphenyl-4,4'-diol (insert, Fig. 6).

The symmetry of the molecule was instrumental in the easy interpretation of the NMR spectra and therefore easy identification of the molecule. In the ^{1}H NMR spectrum three signals in the relative ratio of 6:2:1 were evident in $CDCl_3$. Assignment of the 12-proton singlet at δ 3.94 was for the four methoxy groups at C-2 and C-6, while an aryl doublet (J = 1.8 Hz) at δ 6.68 was assigned to the two *meta* coupled aromatic hydrogens, H-3 and H-5. A broad single peak at δ 6.58 with integration for two hydrogens and D_2O, exchangeable, is assigned to the two phenolic groups at C-1.

The ^{13}C NMR spectrum was equally easy to assign again due its high degree of symmetry. The signal at δ 56.1 was clearly due to the 4 MeO groups while a relatively strong signal at δ 103.8 is assigned to C-3 and C-5 being the only ones with attached hydrogens to increase the relaxation times thus enhancing the intensity of the signal. Weak signals at δ 133.2 and δ 134.4 were assigned to C-4 and C-1, respectively. Finally the signal for C-2 and C-6 at δ 147.1 is the most de-shielded as a consequence of it being attached to methoxy groups.

Based on the results of LC-MS and NMR, the scheme shown in Fig. 7 is proposed as the possible pathway for the synthesis of the dimer. As shown in Fig. 7, the 2,6-DMP went through a single-electron-oxidation by laccase catalysis to produce phenoxy radical species which form

para-radical species through resonance stabilisation. A recombination of two molecules of *para*-radical species then produced the dimer. Our observations are similar to the work of Wan et al., [22] which focussed on characterising the oxidation products as a way of understanding the suitability of 2,6-dimenthoxyphenol as a laccase substrate in different reaction conditions, whereas the current work seeks not only to chemically characterise the dimeric products but also to investigate their potential as antioxidants. In earlier studies, two dimeric products (m/z 304 and m/z 306) were produced in crystal form when the extracellular enzyme activities of *Trametes versicolor* were investigated for their ability to oxidatively couple 2,6-DMP [24]. Comparative experiments using different laccase substrates demonstrated that 2,6-DMP was the most suitable substrate for laccase assays as judged by a number of factors including the stability of its oxidised dimeric coloured product (3,3',5,5'-tetramethoxydiphenylquinone) form, its high absorption molar coefficient, weak acidic optimal pH and oxidation efficiency for a number of blue multicopper enzymes [23]. In related studies, laccase-catalysed coupling of 2,6-DMP in acetone buffer mixture was reported to produce 3,3'5,5'-tetramethoxydiphenolquinone via the C-C coupling in acidic conditions and C-O coupling in basic conditions producing 2,6-dimethyl-1,4-phenylene oxide [37].

3.2. Effect of organic solvents

2,6-DMP is frequently used as a substrate of the laccase oxidation assay and the enzyme activity determination is usually performed in aqueous solution. However, due to inherent advantages of using organic solvents in biocatalysis reactions [38,39], the effect of the nature of the organic solvent, the concentration of the solvent and biocatalysis reaction time were studied in order to obtain the best reaction conditions for product formation. Biocatalysis in the presence

of organic solvents already has been shown to result in the synthesis of novel compounds and opens up new synthetic pathways. Generally, organic solvents have a lot of advantages when employed in biocatalysis, such as higher solubility of hydrophobic species, reduction of water activity, reduction of microbial contamination and the incidence of side reactions found in water, it aids separation and results in improved yields [40].

In the biphasic system, there was an increase in the formation of the major product, P4 (dimer) (as measured by HPLC-MS analysis), as the concentration of ethyl acetate was increased from 50 % to 90 %, after which a decline in product formation was observed (Fig. 8). However, in toluene no product of interest was formed. Production of the dimer in ethyl acetate as co-solvent was increased until 7 h of incubation after which there was a pronounced decline in product formation (Fig. 9).

In the monophasic system, solvents with a lower value of relative polarity favoured the formation of the 2,6-DMP dimer, product P4 (Fig. 10): acetone (0.355) > ethanol (0.654) > methanol (0.762). In previous studies, the nature of the solvent has been shown to affect enzyme activity in biocatalysis with non-polar hydrophobic solvents often providing higher reaction rates than more polar, hydrophilic solvents [41]. In related work, the enhancement of laccase-catalysed oxidation of catechin and epicatechin in less polar organic solvents (as compared to highly polar media), has been reported [42]. These results further show that the enzyme employed for this study has the ability to function in solvents with lower polarity where the essential water layer bound around the enzyme active site has not been stripped away [43].

3.3. Antioxidant activity determination

Many spectrophotometric assays are currently employed to measure the antioxidant capacity of biological samples. The most popular are the ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) or TEAC (trolox equivalent antioxidant capacity) assay, the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, and the FRAP (ferric reducing antioxidant power) assay [44]. Specifically, the ABTS assay is based on the generation of a blue/green $ABTS^{\cdot+}$ that can be reduced by antioxidants; the DPPH assay is based on the reduction of the purple $DPPH^{\cdot}$ to 1,1-diphenyl-2-picryl hydrazine whereas, the FRAP assay is different from these two as there are no free radicals involved, but the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is monitored. These assays are quick and do not require sophisticated equipment, such as a fluorescence detector or a GC-MS, which make these assays suitable for the analyses of multiple tissue samples. The antioxidant activities of the synthesised product (P4) and the substrate (2,6-DMP) were therefore evaluated using these three methods.

Interestingly, the DPPH scavenging activity, TEAC and FRAP analysis of the products showed that the dimer exhibited higher antioxidant activity than the substrate (Table 1). The dimer showed a 119.32, 53.15 and 93.25 % increase in antioxidant activity for FRAP, TEAC and DPPH, respectively, as when compared to the substrate. The increase in antioxidant capacity of the dimer could be attributed to an increase in electron donating groups after dimerisation [45], which tends to reduce the O-H bond dissociation energy and favour the resonance delocalisation of the phenoxyl radical [46].

4. Conclusion

Laccase from *T. pubescens* catalysed the oxidation of 2,6-DMP in a monophasic or biphasic system to form a symmetrical C-C linked dimer with higher antioxidant capacity than the substrate, as was demonstrated by standard antioxidant assays (DPPH, FRAP and TEAC). By appropriate selection of the organic co-solvent, the dimer was obtained in good yields. To the best of our knowledge, this is the first report on laccase-catalysed oxidation of 2,6-DMP to produce potent antioxidants. As antioxidants continue to have value as nutraceuticals and /or components of cosmetics, this compound can find useful application in such areas.

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Table 1

Antioxidant activity of 2,6-dimethoxyphenol (2,6-DMP) and the dimeric product, P4

Molecule	Molecular weight	EC_{50} DPPH[a]	TEAC[b] value	FRAP[c] value
2,6-DMP	154.18	0.802 ± 0.005	1.095 ± 0.006	1.242 ± 0.005
2,6-DMP dimer, product P4	306.11	0.415 ± 0.012	1.677 ± 0.011	2.724 ± 0.045

[a] EC_{50} is defined as the concentration (mM) of antioxidant that brings about 50 % loss of the $DPPH^{\cdot}$ [30]. Values are means ± SD of three replicate determinations.

[b] The Trolox equivalent antioxidant activity (TEAC) of the antioxidant is defined as the concentration of Trolox solution (mM) with an antioxidant potential equivalent to 1.0 mM solution of the substance under investigation [47].

[c] The FRAP (ferric reducing antioxidant power) of the sample is the concentration of ascorbic solution (mM) having the ability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) with an equivalent antioxidant potential to 1.0 mM solution of the sample under investigation [32].

Figure captions

Fig. 1. Calibration curve for the determination of FRAP value using ascorbic acid standards (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM). $y = 1.0707x + 0.0653$, $R^2 = 0.9882$.

Fig. 2. Laccase-catalysed oxidation of 2,6-dimethoxyphenol to form dimeric products (P1-P5)

Fig. 3. Mass spectra of the oxidation products of 2,6-dimethoxyphenol during oxidation by *T. pubescens* laccase.

Fig. 4. MS/MS spectra of the main oxidation product (*m/z* 305.0672, A) of 2,6- dimethoxyphenol and the minor products (*m/z* 277.0726, B; *m/z* 291.0883, C)

Fig. 5. Predicted mechanism of mass fragments formation from the dimer of 2,6-dimethoxyphenol.

Fig. 6. ^{1}H NMR spectrum of the dimer formed during laccase-mediated oxidation of 2,6-dimethoxyphenol

Insert: elucidated structure of the dimer (3,3',5,5'-tetramethoxy biphenyl-4,4'-diol)

Fig. 7. Proposed mechanism for the laccase – catalyzed oxidation of 2,6 – dimethoxyphenol to produce the dimer (3,3',5,5'-tetramethoxy biphenyl-4,4'-diol) .

Fig. 8. Effect of ethyl acetate content on the formation of the 2,6-Dimethoxyphenol dimer (P4) in a biphasic system with sodium acetate buffer (pH 5.0) as co-solvent. All results are means ± standard deviation (SD) of three replicate determinations.

Fig. 9. Effect of reaction time on laccase-catalyzed oxidation of 2,6 dimethoxyphenol. All results are means ± SD of three replicate determinations.

Fig. 10. Effect of organic co-solvent on laccase-catalyzed oxidation of 2,6-dimethoxyphenol to form the dimer, product P4. All results are means ± SD of three replicate determinations.

Fig. 1.

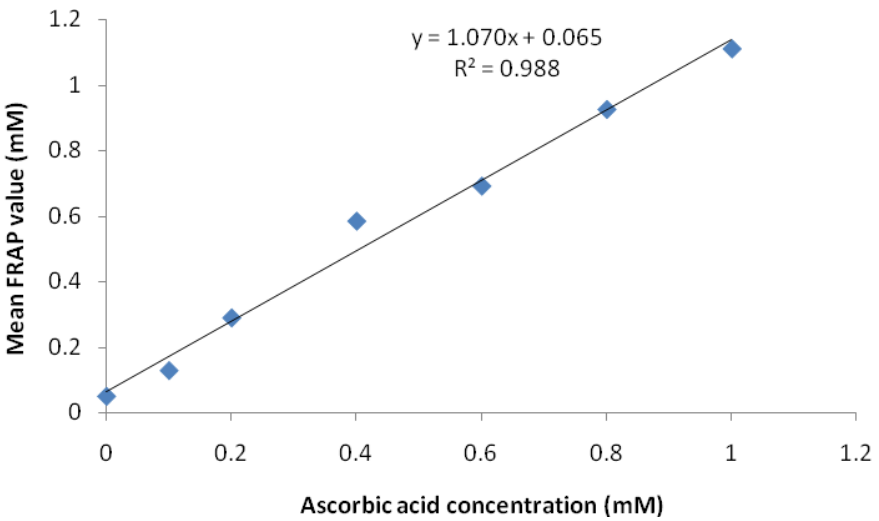

y = 1.070x + 0.065
R² = 0.988
Mean FRAP value (mM)
Ascorbic acid concentration (mM)
0
0.2
0.4
0.6
0.8
1
1.2

Fig. 2.

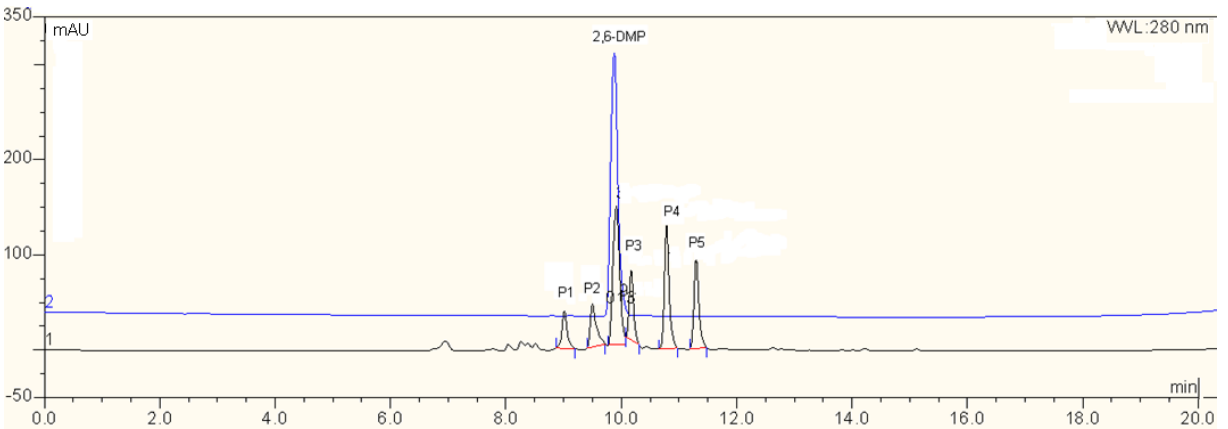

mAU
WVL:280 nm
2,6-DMP
P1
P2
P3
P4
P5
2
1
min
350
200
100
-50
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0

Fig. 3.

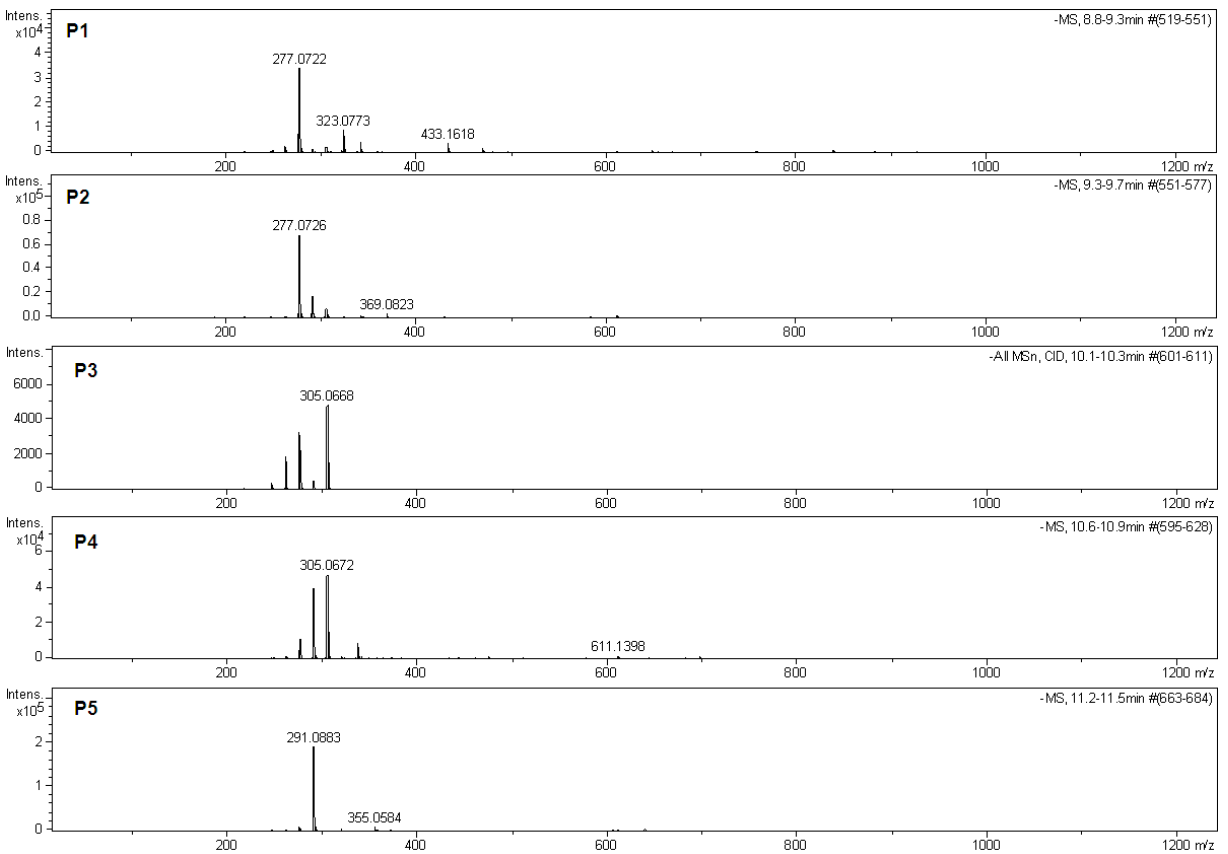

Intens.
x10^4
P1
-MS, 8.8-9.3min #(519-551)
277.0722
323.0773
433.1618
Intens.
x10^5
P2
-MS, 9.3-9.7min #(551-577)
277.0726
369.0823
Intens.
P3
-All MSn, CID, 10.1-10.3min #(601-611)
305.0668
Intens.
x10^4
P4
-MS, 10.6-10.9min #(595-628)
305.0672
611.1398
Intens.
x10^5
P5
-MS, 11.2-11.5min #(663-684)
291.0883
355.0584
200
400
600
800
1000
1200 m/z

Fig. 4.

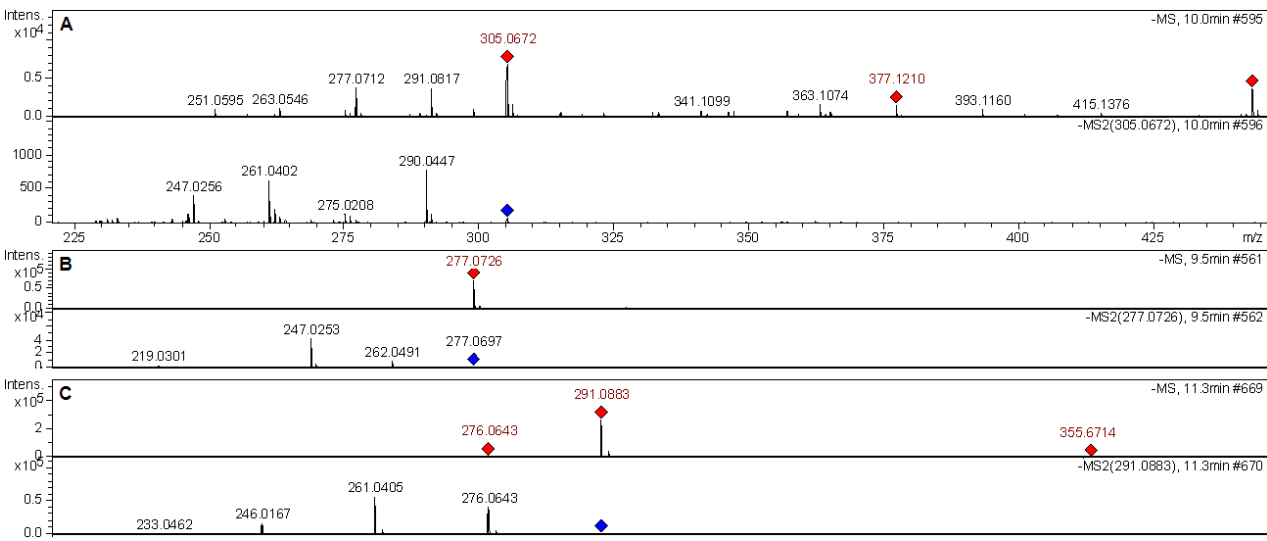

A
-MS, 10.0min #595
305.0672
277.0712
291.0817
251.0595
263.0546
341.1099
363.1074
377.1210
393.1160
415.1376
-MS2(305.0672), 10.0min #596
290.0447
261.0402
247.0256
275.0208
m/z
B
277.0726
-MS, 9.5min #561
-MS2(277.0726), 9.5min #562
247.0253
277.0697
219.0301
262.0491
C
291.0883
276.0643
355.6714
-MS, 11.3min #669
-MS2(291.0883), 11.3min #670
261.0405
276.0643
246.0167
233.0462

Fig. 5.

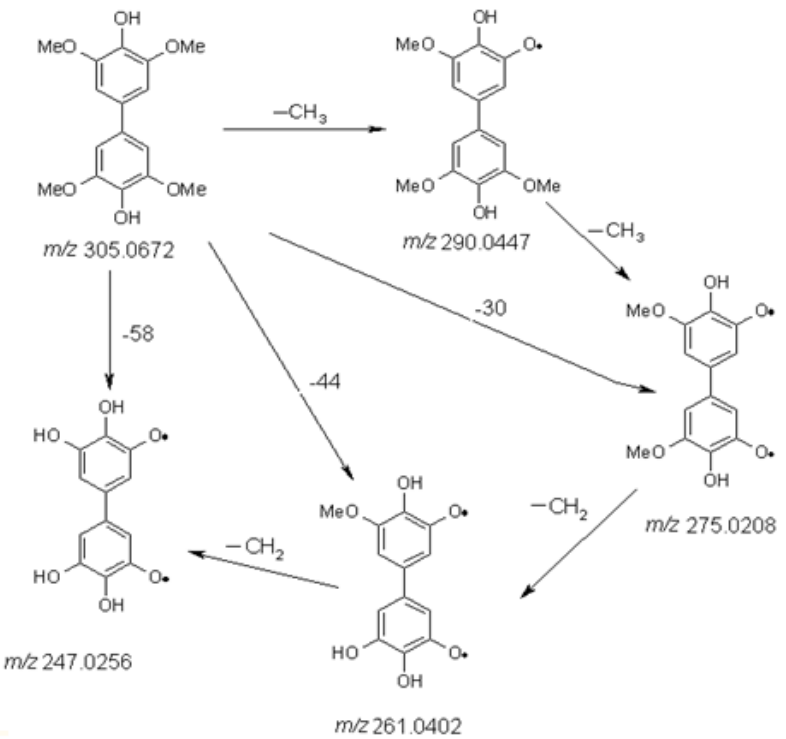

OH
MeO
OMe
MeO
OMe
OH
m/z 305.0672
−CH3
OH
MeO
O•
MeO
OMe
OH
m/z 290.0447
−CH3
-58
-44
-30
OH
HO
O•
HO
O•
OH
m/z 247.0256
−CH2
OH
MeO
O•
HO
O•
OH
m/z 261.0402
−CH2
OH
MeO
O•
MeO
O•
OH
m/z 275.0208

Fig. 6.

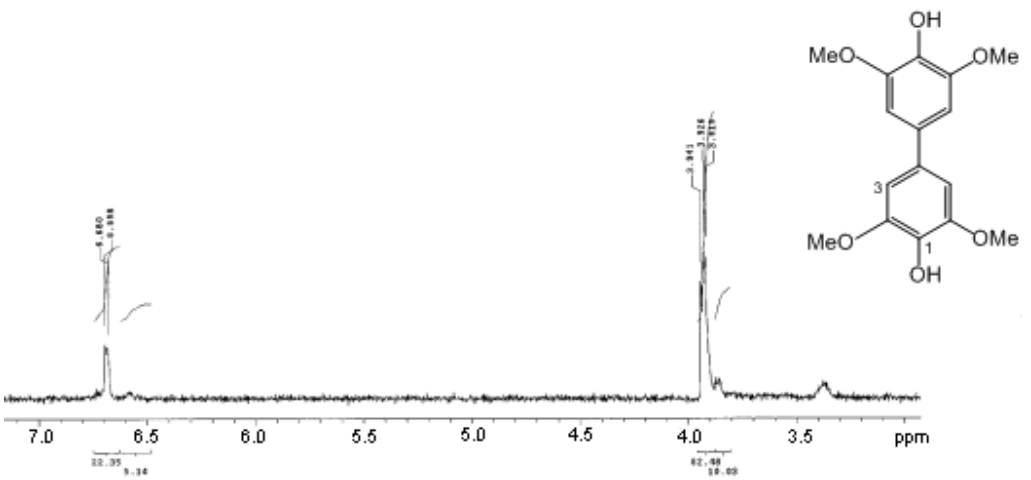


Fig. 7.

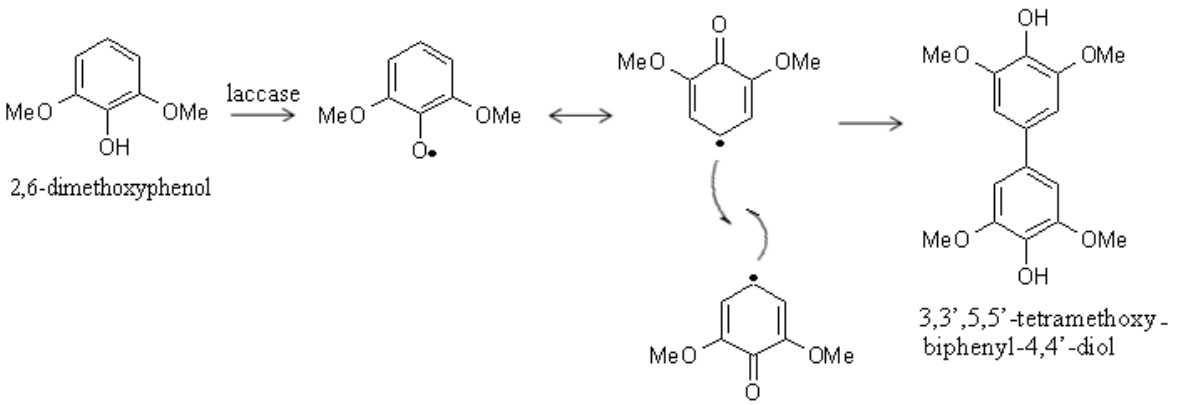

MeO
OMe
OH
2,6-dimethoxyphenol
laccase
MeO
OMe
O•
MeO
OMe
O
MeO
OMe
O
OH
MeO
OMe
MeO
OMe
OH
3,3',5,5'-tetramethoxy-biphenyl-4,4'-diol

Fig. 8.

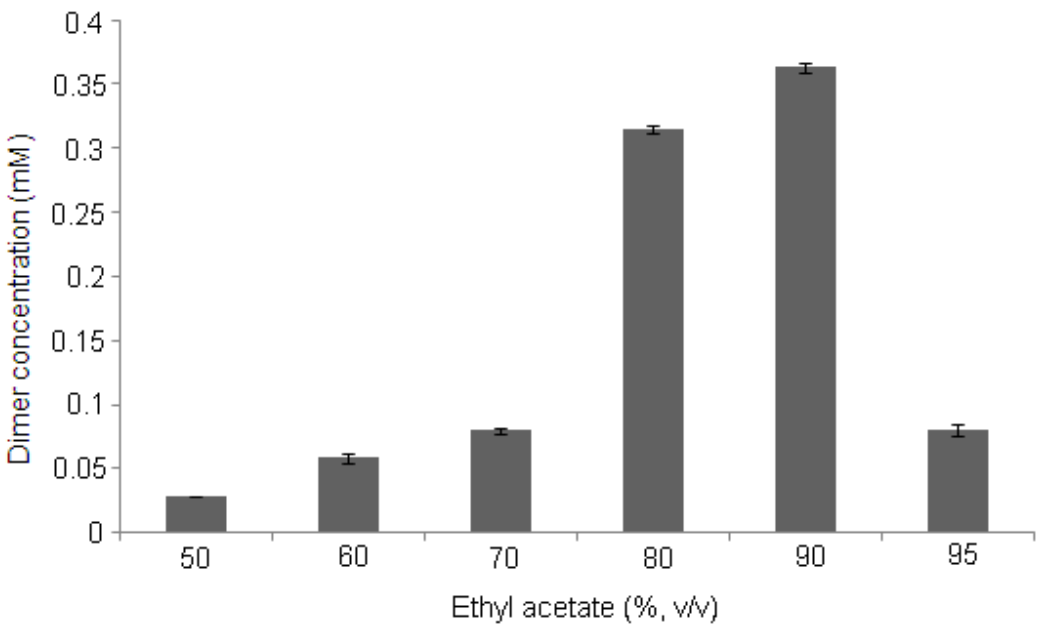

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Dimer concentration (mM)
50
60
70
80
90
95
Ethyl acetate (%, v/v)

Fig. 9.

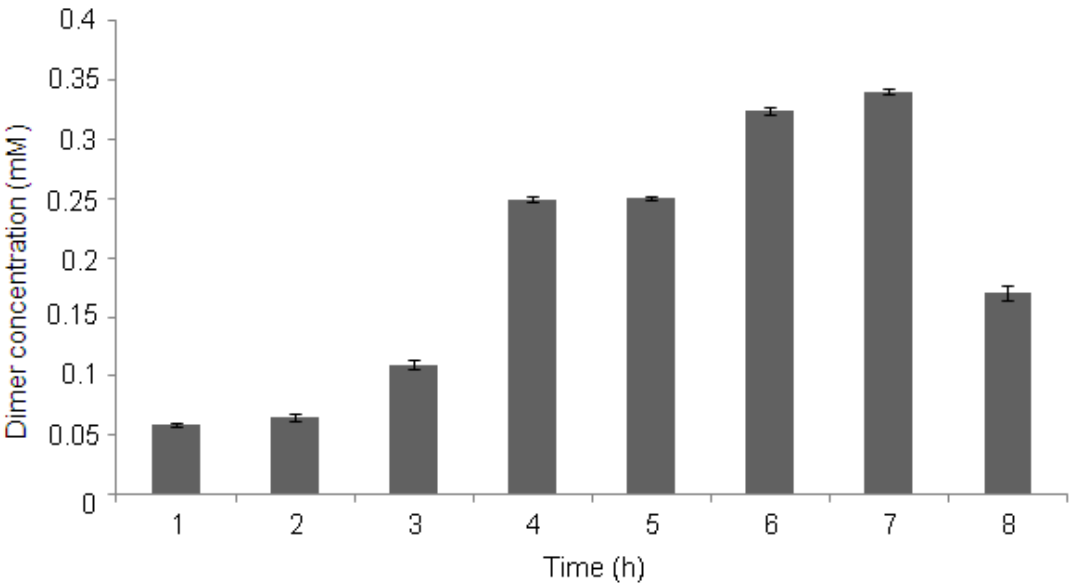
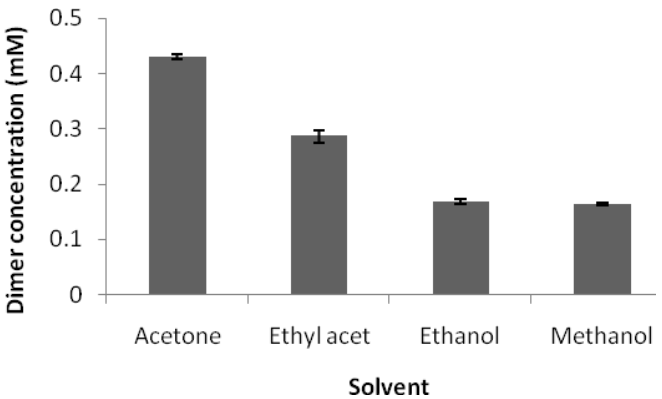

Dimer concentration (mM)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
5
6
7
8
Time (h)

Fig. 10.


Dimer concentration (mM)
0.5
0.4
0.3
0.2
0.1
0
Acetone
Ethyl acet
Ethanol
Methanol
Solvent