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# Purification and characterisation of new laccase from *Trametes polyzona* WRF03

Tobechukwu Christian Ezike*, Arinze Linus Ezugwu, Jerry Okwudili Udeh, Sabinus Oscar Onyebuchi Eze, Ferdinand Chiemeka Chilaka

*Department of Biochemistry, University of Nigeria, Nsukka, Enugu State, Nigeria*

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ABSTRACT

The molecular screening for laccase specific gene sequences in *Trametes polyzona* WRF03 (*Tp*WRF03) using designed oligonucleotide primers analogous to the conserved sequences on the copper-binding regions of known laccases showed positive amplification with an amplicon size corresponding to 1500 bp. The purified *Tp*WRF03 laccase (*Tp*L) is a monomer with a molecular weight corresponding to 66 kDa. The enzyme had an optimal pH of 4.5 and temperature of 55 °C. *Tp*L was most stable within pH of 5.5–6.5 and at a temperature range of 40–50 °C. Sodium azide, sodium cyanide and $Fe^{2+}$ greatly inhibited the enzyme activity. *Tp*L showed more than 50 % decolourisation efficiency on coomassie brilliant blue (72.35 %) and malachite green (57.84 %) but displayed low decolourisation efficiency towards Azure B (1.78 %) and methylene blue (0.38 %). The results showed that *Tp*WRF03 produces high-yield of true laccase with robust properties for biotechnological applications.



## 1. Introduction

Laccase (EC 1.10.3.2) is a multi-copper containing oxidase that catalyzes electron oxidation of phenolic and non-phenolic aromatic substrates with resultant reduction of molecular oxygen to water [1]. Laccase is a monomer and a glycosylated protein with apparent molecular weight from 54 to 97 kDa depending on the source specie [2,3]. It contains four copper atoms (all in +2 oxidation state) distributed to three redox copper centres namely, type-1 copper (mononuclear), type-2 copper and type-3 copper centre. The type-1 copper centre is responsible for the initial oxidation of the substrate while type-2 copper and type-3 copper centres form a trinuclear cluster where the actual catalytic mechanism of laccase occurs [4,5].

Laccase utilizes molecular oxygen as electron acceptor to oxidize its substrates by electron transfer mechanism to unstable free radical intermediates and subsequent breakdown of the compound by non-enzymatic reactions [6,7]. This catalytic property coupled with its broad substrate specificity make laccase applicable to several biotechnological processes such as in pulp bleaching in the paper industry, dye decolourization in dye and textile industry, bioremediation, organic synthesis and biosensor fabrication for detection of polyphenols in wine and juice [4,8]. In addition, laccase has been reported to have anti-proliferative and anti-cancer activity [9].

Laccase activity has been detected in some higher plants, fungi, bacteria, and insect with varied physiological functions and redox potential. However, white-rot fungi basidiomycetes have been reported as the most effective producers of laccase with higher redox potential [10,11]. In white-rot fungi, laccases are encoded by gene families that typically produce multiple isoenzymes [12,13]. It has been proposed that these genes encoding various isoenzymes are differentially regulated with some constitutively expressed and others inducible [14,15]. Consequently, there are numerous isoforms of laccase exhibiting different kinetic and physicochemical properties even within the same fungal strain. These isoforms are induced under different cultivation conditions or expressed at different stages during the fungal life cycle [2,7].

Laccase genes obtained from different white-rot fungi are have been reported to be of different sizes. The size of lac1 gene obtained from *Lentinula edodes* was about 1.5–1.6 Kb [16]. Okamoto et al. [17] reported a laccase gene of 1.59 Kb in size from *Pleurotus ostreatus* lccK. Although different white rot fungi have laccase genes of various sizes and different number of isoforms, they exhibit a similar pattern in their sequences. The ten histidine residues and one cysteine residue that coordinated the four catalytic cupric ions are well conserved in laccase, including a few number of sequence close to the region where the four copper ligands are clustered [18]. The amino acid sequences around these conserved regions include; Cu I (HWHGFFQ), Cu II (HSHLSTQ), Cu

* Corresponding author.
*E-mail address:* tobechukwu.ezike@unn.edu.ng (T.C. Ezike).

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III (HPFHLHGH), and Cu IV (HCHIDWHL) [19]. Souza et al. [20] proposed the use of designed oligonucleotide primers corresponding to these conserved sequences for PCR amplification of laccase genes. In addition, previous works carried out with primer pair designed from these conserved sequences showed positive results and were recommended for sensitive detection of laccase gene-specific sequences in white-rot fungi [21–23]

The present study was therefore, to evaluate the kinetics and biochemical properties of the major isoform of laccase secreted by autochthonous WRF, *Trametes polyzona* WRF03 for biotechnological applications. Designed oligonucleotide primer pair corresponding to the conserved sequences of Cu I and Cu IV binding regions of known laccase gene was first used to screen for laccase gene-specific sequences from the fungus prior to characterisation. The screening strategy adopted is to ensure that the fungus produces true laccase since other groups of phenol oxidase such as tyrosinases and catechol oxidases catalyze overlapping substrates with laccase [24].

## 2. Materials and methods

### 2.1. Chemicals and agro-waste

Major chemicals such as 2,2' azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), Sephadex G-100, Diethylamino ethyl Cellulose (DEAE-cellulose), bovine serum albumin (BSA) and Folinciocalteau reagents were obtained from Sigma-Aldrich, Germany while malt extract agar (MAE) and potato dextrose agar (PDA) were obtained from JHD, China. Wheat bran was procured from a nearby market in Nsukka, Enugu State, Nigeria and ground to a particle size of 1 mm. The powdered wheat bran was stored in a dry container and was used as both an inducer and lignocellulosic support.

### 2.2. Microorganism and inoculum

A hypersecretory white-rot fungi strain, *Trametes* polyzona WRF03 obtained from the Microbial Culture Collection in Enzymology and Protein Chemistry Unit (MCCEPU), Department of Biochemistry, University of Nigeria, Nsukka, maintained on PDA slant at 4 °C, and sub-cultured every 30 days (periodic transfer). Prior to the experiment, the organism was sub-cultured on Petri dishes using the same medium and used as inoculum.

### 2.3. Fermentation for laccase production

Fermentation was done in Erlenmeyer flask (250 ml) containing 20 ml of modified basal medium proposed by Tien and Kirk [25] under stationary condition. The basal medium contained (g/L); ammonium tartarate (0.22), $KH_2PO_4$ (0.2), $MgSO_4.7H_2O$ (0.05), Thiamine (0.001), $CuSO_4$ (0.08), $CaCl_2$ (0.01), $NaMO_4$ (0.05), $FeSO_4$ (0.03), $MnSO_4$ (0.07), glucose (10) and wheat bran (5). Each flask was inoculated with three (3) plugs of one (1) cm diameter cut from the growing PDA pure culture *Trametes polyzona* WRF03 and incubated at 25 °C for twelve (15) days. The content of duplicate flasks was harvested after each day and the laccase activity was determined.

### 2.4. Assay for laccase activity and protein content determination

Laccase activity was determined by little modification of the method of Shin and Lee [26] using ABTS as substrate. The reaction mixture contained 2.8 ml of sodium acetate buffer (0.1 M, pH 4.5), 0.1 ml of 1 mM ABTS and 0.1 ml of the enzyme. The oxidation of ABTS was observed at 420 nm ($\varepsilon_{420} = 36\ mM^{-1}\ cm^{-1}$) by measuring the change in absorbance for 1 min. The protein content was measured by Lowry et al. [27] method using Bovine serum albumin (BSA) as a standard protein.

### 2.5. Screening for laccase-gene specific sequence

The mycelia of *Trametes polyzona* WRF03 was harvested from the solid culture at the peak of laccase activity. Genomic DNA (gDNA) was extracted using the *AccuPrep*® DNA extraction kit (Bioneer, Korea). Designed oligonucleotide primers corresponding to conserved sequences around Cu I (5'-CACTGGCACGGCTTCTTCCA-3`) and Cu IV (5' – TGGCAGTGGAGGAACCACGG - 3') coding for HWHGFFQ and PWFLHCH in laccase genes were used as forward and reverse primers respectively. PCR was performed using the gDNA of *Trametes polyzona* WRF03 as template with a predenaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 57 °C), and extension (30 s at 72 °C) and then by a final incubation (5 min at 72 °C). Agarose gel electrophoresis was performed on the PCR products using 1.5 % (w/v) agarose powder (QD LE Agarose- Green Biore) in 1 X TAE (40 mM Tris-acetate in 1 mM EDTA, pH 8.0) buffer. The amplicons were visualized using ChemiDoc™ MP Imaging System (Bio-Rad, USA).

### 2.6. Purification of laccase

#### 2.6.1. Ammonium sulphate precipitation

Ammonium sulphate was added slowly to the crude enzyme with gentle stirring to attain 90 % saturation. The mixture was kept for 30 h at 4 °C and then spun with a centrifuge at 10,000 rpm for 10 min at 4 °C. The pellet obtained was dissolved in a little volume of sodium phosphate buffer (0.1 M, pH 7.5) and laccase activity assayed. The protein solution was dialyzed against sodium phosphate buffer (0.01 M, pH 7.5) for 12 h with buffer replacement every 6 h. Laccase activity and the total protein content of the dialysate was assayed as previously described.

#### 2.6.2. Ion-exchange chromatography (IEC)

A volume (10 ml) of the dialysate was introduced to a DEAE-cellulose column (2 × 30 cm) previously equilibrated with sodium phosphate buffer (0.01 M, pH 7.5). The column was washed with 50 ml of the same buffer followed by stepwise elution of bound proteins with NaCl solution (0.1 – 0.5 M) prepared with the equilibration buffer. All chromatographic fractions were collected in 5 ml fractions at a flow rate of 2.5 ml/min and assayed for laccase activity. Fractions rich in laccase activity were combined and concentrated for the next purification step.

#### 2.6.3. Gel filtration chromatography

A volume (10 ml) of the concentrated enzyme obtained after ion-exchange chromatography was carefully applied into Sephadex G-100 column (2 × 76 cm) equilibrated with sodium phosphate buffer (0.1 M, pH 7.5). Fractions of 5 ml were collected at a flow rate of 0.25 ml/min and then assayed for laccase activity. The protein content of each fraction was measured at 280 nm.

#### 2.6.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

After gel filtration, an aliquot of the pooled fractions with highest laccase activity was subjected to SDS-PAGE analysis to confirm the purity of the enzyme and to determine the sub-unit molecular weight of the purified laccase from *Tp*WRF03. The SDS-PAGE was carried out using *AccuLadder*™ Protein size marker (Broad) (Bioneer, USA) containing β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor

(20.1 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa) by following the manufacturer's instruction.

### 2.7. Characterisation of laccase

#### 2.7.1. Effect of pH on laccase activity and stability

Laccase activity was assayed at different pH ranging from 3.0–10.0 using 0.1 M sodium acetate buffer (pH 3.5–5.5), phosphate buffer (pH 6.0–7.5) and Tris- HCl buffer (pH 8.0–9.5) at pH interval of 0.5. The stability of the enzyme was tested in a pH range of 3.0–10.0 by incubating the enzyme at different pH values for 120 min at room temperature and the residual enzyme activity was assayed at a regular time interval using ABTS.

$$\%\,\text{Residual Activity (RA)} = \frac{\text{Enzyme activity after pH treatment}}{\text{Enzyme activity before pH treatment}} \text{x } 100$$

#### 2.7.2. Effect of temperature on laccase activity and stability

The effect of temperature on laccase activity was tested at different temperatures ranging from 30 to 100 °C (5 °C interval) at the optimum pH. A mixture of the substrate (ABTS) and buffer (0.1 M sodium acetate, pH 4.5) were incubated in a water bath for 10 min at different temperature for equilibration before the enzyme was added to start the reaction. The thermal stability of the enzyme was investigated by incubating laccase at varying temperatures (40–70 °C) at the optimum pH for regular time interval over 120 min period water bath (Model DK-420, China). Following heating, aliquots of the enzyme were withdrawn at regular time intervals, cooled on ice and residual activity were assayed. The stability of the enzyme was expressed as percentage residual activity (% RA).

#### 2.7.3. Kinetic parameters of the purified TpL

Kinetic study of laccase-catalyzed oxidation of ABTS at different concentrations (10–200 μM) was carried out at pH 4.5 and 25 °C to determine kinetic constants, $V_{max}$ and $K_m$. The kinetic constants were calculated from Lineweaver-Bulk plot of the reciprocal of initial velocities and substrate concentrations.

#### 2.7.4. Effect of organic solvent on laccase stability

The organic solvent-tolerance of laccase was examined. This was done by incubating the enzyme in various organic solvents, such as acetonitrile, acetone, ethanol, methanol, and propanol at a final concentration of 10, 20, 30 and 40 % (v/v) for 1 h according to Huang et al. [28]. The enzyme activity was determined as previously described.

#### 2.7.5. Effect of metal ions on laccase activity

The effect of several metal ions including $Mg^{2+}(MgSO_4.7H_2O)$, $Cu^{2+}(CuSO_4)$, $Ba^{2+}$ ($BaCl_2$), $Zn^{2+}(ZnCl_2)$, $Ca^{2+}(CaCl_2)$, $Pb^{2+}(PbCl_2)$, $Mn^{2+}(MnCl_2.4H_2O)$, $Hg^{2+}(HgCl_2)$, $Co^{2+}(CoCl_2)$, and $Fe^{2+}(FeSO_4)$ on laccase activity was investigated [6]. Metal ions concentration varied from 10 to 50 mM in sodium acetate buffer (0.1 M, pH 4.5) and 0.1 ml of the enzyme. The reaction was initiated by adding the substrate (ABTS) and the residual activity was determined.

#### 2.7.6. Effect of inhibitors on laccase activity

The following organic compounds such as chelating agent (EDTA), sodium azide ($NaN_3$), amino acid (cysteine), urea, sodium deodocyl sulphate (SDS) and sodium cyanide were evaluated for their inhibitory effect on the enzyme activity. The inhibitory action on the enzyme was tested by pre-incubation of the enzyme with the inhibitors at different concentrations (1 mM–10 mM) for 5 min to ensure complete inhibition [29].

$$\%\,\text{Inhibition} = \frac{A_{CT} - A_{IT}}{A_{CT}} \text{x } 100$$

Where, $A_{IT}$ = absorbance on inhibition test after 1 min and $A_{CT}$ = absorbance in control test after 1 min)

#### 2.7.7. Spectroscopic studies on Trametes polyzona WRF03

The UV–vis absorption spectrum of the purified enzyme in sodium acetate buffer (0.1 M, pH 4.5) was measured at room temperature (27 °C) using UV–vis spectrophotometer (Jenway 6405) in 1 cm path length UV quartz glass cell [6].

#### 2.7.8. In-vitro studies on decolourisation of synthetic dyes

The ability of the enzyme to decolourize seven (7) synthetic dyes as coomassie brilliant blue, malachite green, methyl orange, eriochrome black, congo red, azure B and methylene blue was examined. A volume of 0.1 ml of the stock solution (200 mg/mL) of different dyes was added to 2.4 ml of sodium acetate buffer (pH 4.5) in a separate tube followed by the addition of 0.5 ml of purified enzyme solution. The percentage reduction of the colour was monitored spectrophotometrically for 6 h at the characteristic wavelength of each dye [30]. A decrease in absorbance shows that there was decolourisation. The decolourisation efficiency (%) was calculated as follows.

$$\%\,\text{Decolourisation} = \frac{A_\lambda initial - A_\lambda final}{A_\lambda initial} \text{x } 100$$

Where, $A_\lambda initial$ = initial absorbance of the untreated dye at their characteristic peak and $A_\lambda final$ = absorbance after treatment with laccase at the same peak per unit time

## 3. Results

### 3.1. Production and purification Trametes polyzona WRF03 laccase

The maximum laccase production from *Tp*WRF03 was obtained on the end of 9th day of fermentation with a specific activity of 1637 U/mg protein (Table 1). The crude laccase was precipitated with 90 % ammonium sulphate saturation resulting to specific activity (4262 U/mg protein) and purification fold of 2.60. The dialysate was purified on ion-exchange chromatography (DEAE-cellulose) and one distinctive peak was obtained after elution with 0.1 – 0.16 M NaCl in 0.01 M sodium phosphate buffer pH 7.5 (Fig. 1). The pooled fractions showed a specific activity of 9700 U/mg protein and purification fold of 5.92 (Table 1). The fraction was further purified by gel filtration on Sephadex G-100 and a single peak was obtained. In addition, ultraviolet absorbance at 280 nm of the eluted fractions also showed a single characteristic peak

**Table 1**
Purification profile of laccase from *Trametes polyzona* WRF03.

| Purification step | Total activity (U) | Total Protein (mg) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude Enzyme | 605758 | 369.953 | 1637 | 1.00 | 100 |
| $(NH_4)_2SO_4$ precipitation (90 %) | 564149 | 132.378 | 4262 | 2.60 | 93.10 |
| Dialysis | 443298 | 74.611 | 5941 | 3.63 | 73.20 |
| Ion-exchange (DEAE-Cellulose) | 86066 | 8.873 | 9700 | 5.92 | 14.20 |
| Gel Filtration (Sephadex G-100) | 16993 | 0.790 | 21523 | 13.14 | 2.80 |

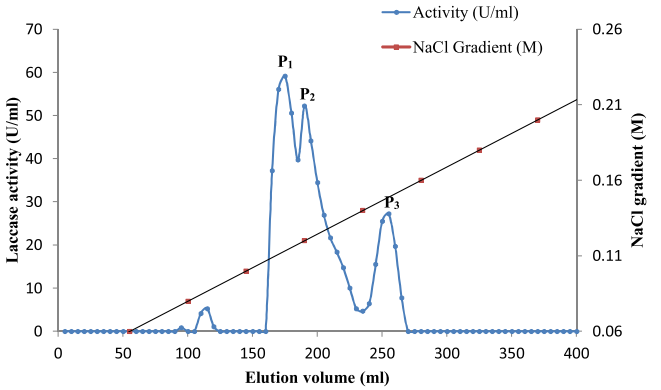


**Fig. 1.** Elution profile of laccase from *Trametes polyzona* WRF03 after ion-exchange chromatography using DEAE-cellulose. The linear sodium chloride gradient was formed from 50 ml of 0.06 – 0.2 M sodium chloride dissolved in sodium phosphate buffer (0.01 M pH 7.5). Fraction volumes of 5 ml were collected at a flow rate of 2.5 ml/min.

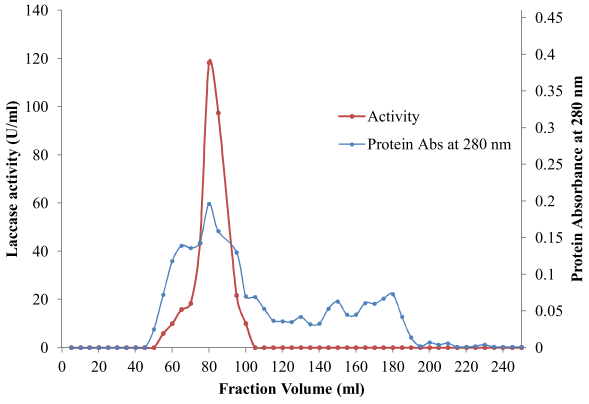


**Fig. 2.** Elution profile of laccase from *Trametes polyzona* WRF03 after gel filtration using Sephadex G-100. The column was equilibrated and eluted with sodium phosphate buffer (0.1 M, pH 7.5). Five millilitre (5 ml) fractions were collected at a flow rate of 0.25 ml/min at 25 °C.

(Fig. 2). After gel filtration, laccase from *Trametes polyzona* WRF03 (*Tp*L) exhibited a specific activity of 21523 U/mg protein towards ABTS at the standard assay condition with a percentage yield of 2.8 and overall 13-fold purification (Table 1). The purified *Tp*L showed a single protein band at approximately 66 kDa as shown in Fig. 4. Spectra scan of purified *Tp*WRF03 laccase showed no characteristic peak at 610 nm (Fig. 9).

### 3.2. PCR amplification of laccase gene-specific sequence

The gene-specific primers used for gDNA amplification of laccase gene showed positive amplification. An amplicon size within 1500–1600 bp was observed on the agarose gel electrophoretogram (Fig. 3).

### 3.3. Effect of pH on Tp*L* activity and stability

The enzyme showed activity over a broad pH range of 3.0–6.5 with maximum activity at pH 4.5 (Fig. 5). In addition, it exhibited higher activity in the acidic region than in the neutral/alkaline region. At pH value higher than 6.5, laccase activity was not

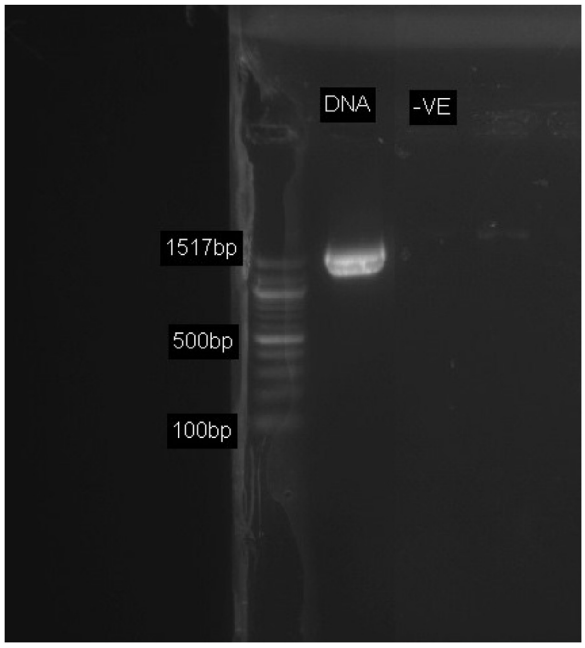


**Fig. 3.** Gel image of PCR products of amplified gDNA of laccase gene. Lanes 1, 2, and 3 represent DNA molecular weight marker, amplified gDNA region of *Tp*WRF03 and negative control respectively.

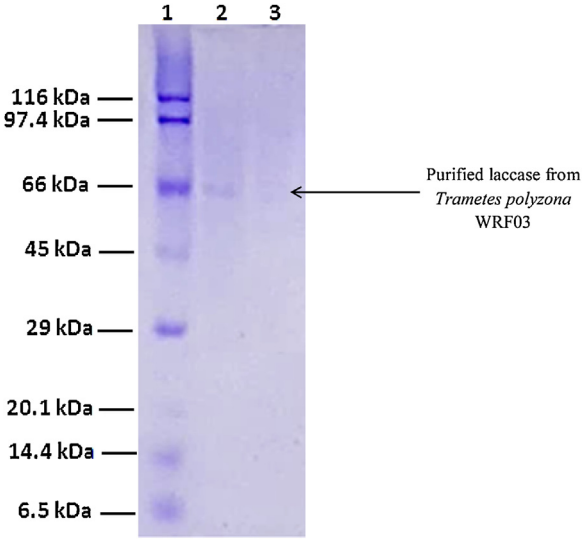


**Fig. 4.** SDS-PAGE of the purified laccase from *Trametes polyzona* WRF03 after gel filtration on Sephadex G-100. All protein bands were stained with Coomassie Blue R-250. Lane 1 is for molecular weight markers whereas lane 2 and 3 are for purified laccase.

detected. Conversely, the enzyme was more stable at higher pH value (in the neutral and alkaline region) than in acidic regions. At pH value of 5.5, 6.0 and 6.5 after 120 min, the enzyme retained about 82 %, 100 % and 93 %, respectively (Fig. 6).

### 3.4. Effect of temperature on Tp*L* activity and stability

The enzyme was active over a wide range of temperatures from 25 °C to 100 °C with optimum activity at 55 °C (Fig. 7). Interestingly, *Tp*L showed detectable activity at 100 °C. However, the enzyme was more stable at 40 and 50 °C and retained 64.38 and 42.92 of its activity respectively, after 120 min (Fig. 8).

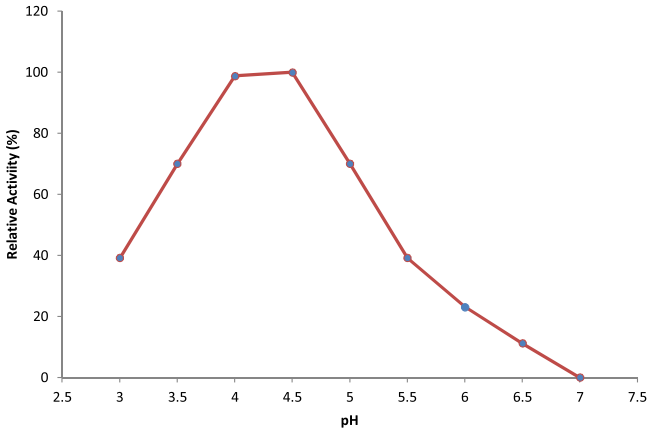


**Fig. 5.** Effect of pH on *Tp*L activity using 0.1 M sodium acetate buffer (pH 3.0 – 5.5), 0.1 M sodium phosphate buffer (pH 6.0 – 7.5) and 0.1 M Tris-HCl buffer (pH 8.0 – 10.0). *Tp*L activity expressed in percentage relative to the optimum pH.

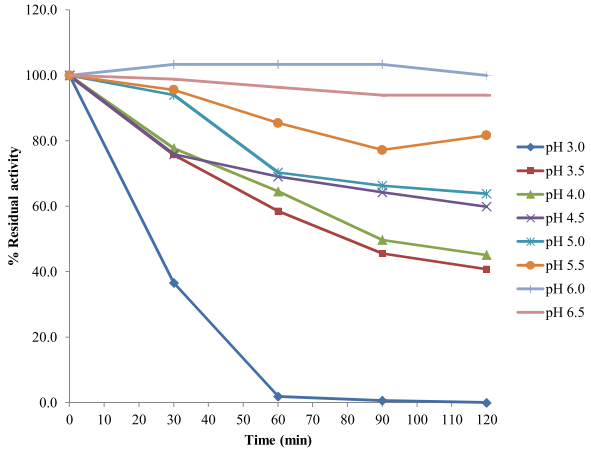


**Fig. 6.** pH stability of laccase from *Trametes polyzona* WRF03 using ABTS as substrate.

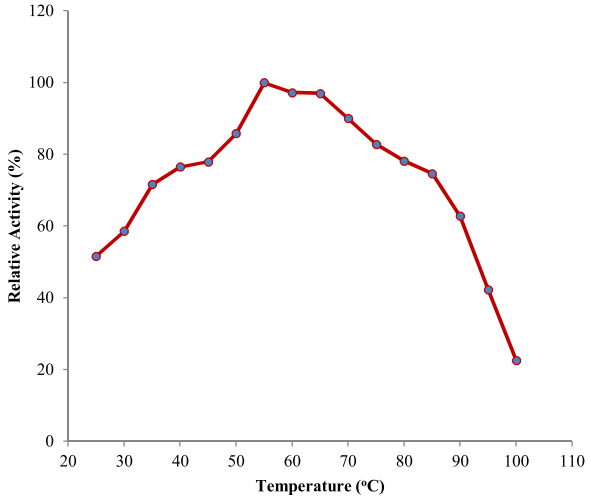


**Fig. 7.** Effect of temperature *Tp*L activity using ABTS as substrate in sodium acetate buffer (0.1 M, pH 4.5). *Tp*L activity expressed in percentage relative to the optimum temperature.

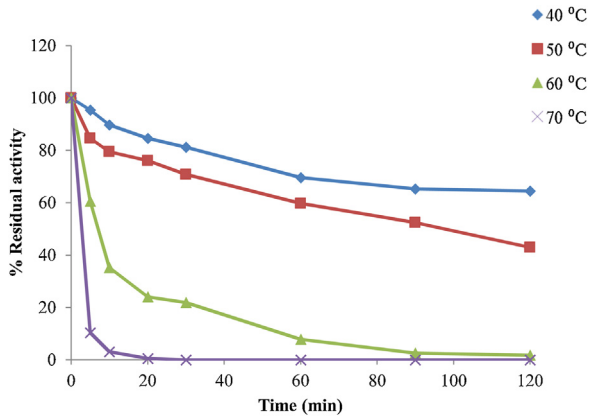


**Fig. 8.** %Residual activity of laccase from *Trametes polyzona* WRF03 at pH 4.5 and temperature of 40, 50, 60 and 70 °C.

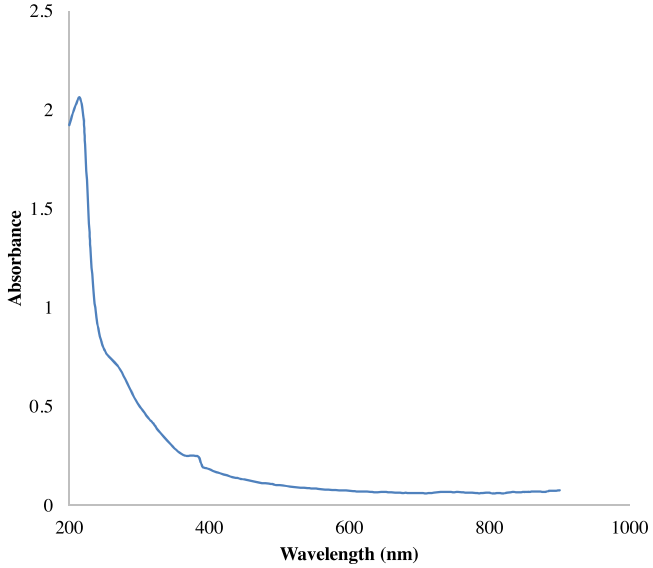


**Fig. 9.** Absorption spectrum of the purified *Tp*L in 0.1 M Sodium acetate buffer (pH 4.5).

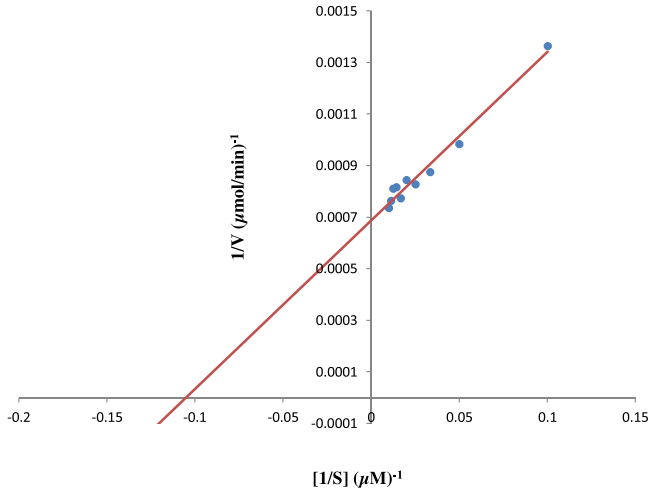


**Fig. 10.** Lineweaver-Bulk plot of *Tp*L catalyzed oxidation of ABTS.

**Table 2**
Organic solvent stability of laccase from *Trametes polyzona* WRF03.

| | Relative laccase activity (%)[a] | | |
|---|---|---|---|
| Organic solvent | 10 mM | 30 mM | 50 mM |
| Control | 100 | 100 | 100 |
| Acetone | 89.68 | 77.59 | 50.00 |
| Acetonitrile | 93.38 | 84.73 | 31.18 |
| Ethanol | 92.55 | 83.65 | 14.55 |
| Methanol | 94.31 | 90.53 | 45.65 |
| Propanol | 92.89 | 72.39 | 41.57 |

[a] Values represent percentage relative to the control. Laccase activity in the absence of organic solvent (control) was regarded as 100 %.

**Table 3**
Effect of divalent metal ion on laccase activity.

| | Relative activity (%)[a] | | |
|---|---|---|---|
| Metal ion | 10 mM | 30 mM | 50 mM |
| Control | 100 | 100 | 100 |
| $Mg^{2+}$ | 77.38 | 95.93 | 105.4 |
| $Cu^{2+}$ | 76.47 | 74.66 | 96.83 |
| $Ba^{2+}$ | 74.21 | 33.48 | 5.88 |
| $Zn^{2+}$ | 49.77 | 23.98 | 3.17 |
| $Ca^{2+}$ | 45.25 | 15.38 | 9.50 |
| $Pb^{2+}$ | 43.89 | 21.72 | 18.55 |
| $Mn^{2+}$ | 27.15 | 12.67 | 0.00 |
| $Hg^{2+}$ | 19.46 | 16.74 | 11.31 |
| $Co^{2+}$ | 0.90 | 19.91 | 7.69 |
| $Fe^{2+}$ | 2.26 | 0.45 | 0.00 |

[a] Values represent percentage relative to the control. Laccase activity in the absence of metal.
ion (control) was regarded as 100 %.

**Table 4**
Effect of putative inhibitors on laccase activity.

| | %Inhibition of laccase activity[a] | | |
|---|---|---|---|
| Inhibitor | 1 mM | 5 mM | 10 mM |
| Sodium cyanide | 91.40 | 96.77 | 100.00 |
| Sodium azide | 81.18 | 91.40 | 100.00 |
| SDS | 83.33 | 88.71 | 89.25 |
| Urea | 45.69 | 57.00 | 65.05 |
| L-Cysteine | 65.05 | 68.82 | 68.28 |
| EDTA | 74.20 | 83.33 | 91.94 |

[a] % Inhibition = $100(A_{CT} - A_{IT})/ A_{CT}$ where, $A_{IT}$ = Absorbance on inhibition test after 1 min and $A_{CT}$= Observed absorbance in control test after 1 min.

### 3.5. Kinetic parameters

The values of the kinetic parameters ($K_m$ and $V_{max}$) of the purified *Tp*L calculated from Lineweaver-Bulk plot (Fig. 10) were 8.66 μM and 1429 μmol/min respectively using ABTS as substrate.

### 3.6. Organic solvent stability

In general, as the organic solvent concentration was increased from 10 to 50 %, the relative activity of *Tp*L decreased, although, the enzyme showed most stability when incubated in 10 % (v/v) organic solvents (Table 2). At 10 % concentration of the organic solvent, the enzyme retained more than 94 %, 93.38 %, 92.55 %, 92.89 % and 89.68 % activity in methanol, acetonitrile, ethanol, propanol and acetone, respectively. The magnitude of decrease in residual activity after each successive increase (10 %) in polar organic solvent was more in propanol and ethanol. At a concentration of 50 % (v/v), the enzyme lost about 85 % activity in ethanol after 1 h. Interestingly, the enzyme showed considerable stability in acetone than other organic solvents and maintained 50 % of its residual activity at 50 % (v/v) concentration.

### 3.7. Effect of metal ions on Tp*L* activity

Some metal ions were tested to determine their effect on *Tp*L. As shown in Table 3, an increase in metal ions concentration (10–50 mM) gradually inhibited the enzyme activity except for $Mg^{2+}$ and $Cu^{2+}$ that showed a slightly stimulatory effect on the enzyme activity. $Fe^{2+}$ was the most potent inhibitor of laccase, causing more than 90 % reduction in enzyme activity at 10 mM. In addition, the enzyme lost 100 % its activity at 50 mM concentration of $Mn^{2+}$.

### 3.8. Effect of putative inhibitors on Tp*L* activity

The inhibition studies revealed that all the inhibitors showed more than 50 % inhibition at 10 mM (Table 4). It was also observed that as the inhibitor concentration was increased from 1 mM to 10 mM, their percentage inhibition also increased. Sodium azide and sodium cyanide showed 100 % inhibition of the enzyme activity at 10 mM.

### 3.9. Dye decolourisation efficiency of Tp*L*

The *Tp*L decolourised all the tested dyes although with different percentage efficiency (Table 5). The enzyme showed more than 50 % decolourisation efficiency on coomassie brilliant blue (72.35 %) and malachite green (57.84 %) but displayed low efficiency towards Azure B (1.78 %) and Methylene blue (0.38 %).

## 4. Discussion

The use of laccase gene-specific primers complementary to the conserved sequences in the cupric ions binding regions of known laccase facilitated rapid amplification of the laccase gene in *Trametes polyzona* WRF03 (*Tp*WRF03). The positive amplification indicated the presence of laccase coding gene in *Tp*WRF03 genome. In addition, the amplicon size falls within the range of expected

**Table 5**
Decolourisation efficiency of laccase from *Trametes polyzona* WRF03 towards different classes of synthetic dye.

| | Type of Dye | Maximum Absorbance[a] | Class of dye | Decolourisation Efficiency (%)[b] |
|---|---|---|---|---|
| 1 | Coomassie brilliant blue | 585 | Triphenylmethane | 72.35 |
| 2 | Malachite green | 615 | Trarylmethane | 57.84 |
| 3 | Methyl orange | 410 | Azo | 47.55 |
| 4 | Erichrome black | 510 | Azo | 40.20 |
| 5 | Congo red | 480 | Diazo | 18.11 |
| 6 | Azure B | 595 | Thiazin | 1.78 |
| 7 | Methylene blue | 650 | Heterocyclic | 0.38 |

[a]The wavelength of maximum absorption of each dye was determined by spectral scan of the respective dyes in the presence of laccase. [b]Dye decolourisation (%) = ($A_\lambda$ initial – $A_\lambda$ final) / $A_\lambda$ initial x100 Where, $A_\lambda$ initial = initial absorbance of the untreated dye at their characteristic peak and $A_\lambda$ final = absorbance after treatment with laccase at the same peak per unit time.

amplicon size (1500–1700 bp) of fungal laccases as reported by many authors that used similar primers [21–23]. The extracellular nature of laccase from *Trametes polyzona* WRF03 and the fact that the enzyme is the only ligninolytic enzyme noticeably produced by the organism facilitated the purification process. The 90 % ammonium sulphate saturation required for maximum precipitation of laccase from solution in this study is in agreement to the report of Chaurasia et al. [31] for laccase from *Trametes hirsute* MTCC-1171. However, Zhu et al. [32], Olajuyigbe and Fatokun [33] reported 80 % ammonium sulphate saturation for laccase from *Russula virescens*, *Trametes trogii* and *Sporothrix carnis* CPF-05, respectively.

Ion-exchange column chromatography (DEAE-Cellulose) played an important role in removing the brown colour pigment from the enzyme solution. The purification process was analogous to that employed for laccase from *Pleurotus sajor-caju* [34]. Multiple peaks obtained were indicative of the presence of more than one form of the protein produced by the organism. Desai and Nityanand [35] stated that laccase just like other ligninolytic enzymes is coded by gene families that allow multiple expression of isoforms, which are regulated at the level of gene expression. However, numerous isoforms can be variants of the same gene resulting from post-transcriptional modifications [36]. Thus, the number of isoforms produced is dependent on the organism and the component of the production medium [37,38]. Zapata-castillo et al. [39] also observed two major peaks denoted as *Lac1* and *LacII* for laccases from *Trametes hirsute* bm-2 on Q-Sepharose exchanger while Ning et al. [40] observed three major peaks (D1, D2 and D3) eluted with 100, 300, and 1000 mM NaCl in Tris-HCl buffer (10 mM, pH 7.2) respectively for laccase from the fungus *Leucoagaricus naucinus*. Many of these isoforms have been carefully separated, purified and characterized [41]. Further purification by gel filtration gave a single peak indicating the presence of a single protein. After gel filtration, *Tp*WRF03 laccase was purified 13-fold with a specific activity of 21523 U/mg protein and 2.8 % recovery. The small fold in the purification factor obtained in the study implies that laccase was mostly expressed by the organism and represents a major protein in the extracellular fluids. Since only one isoform of the enzyme richer in laccase activity after anion exchange chromatography was further purified, the removal of other isoforms may also have contributed to low purification factor and percentage recovery. The result obtained in the present study is comparable to the work of Mansur et al. [14], Sahay et al. [34] and Chaurasia et al. [31] that utilized similar purification steps. The molecular weight of 66 kDa obtained in this study is the same with that of laccase from *Trametes versicolor* [42] and comparable with a molecular weight of 65 kDa and 71 kDa for laccases from *Trametes* sp. LS-10C [43] and *Trametes polyzona* KU-RNW027 [44]. Molecular weights of most fungal laccases have been reported to fall within the range of 60–90 kDa of which 10–25 % may be attributed to the degree glycosylation [45].

The pH optimum of 4.5 for laccase from *Trametes polyzona* WRF03 corresponds to that obtained for laccase from *Trametes polyzona* KU-RNW027 [44], *Trichoderma harzanium* [6], *Pleurotus* sp. [45] and *Trametes hirsuta* [31]. However, Chairin et al. [46] reported a pH optimum of 2.2 for laccase from *Trametes polyzona* WR710–1, while Asgher et al. [47] obtained an optimum pH of 5.0 from *Trametes versicolor* IBL-04 laccase. The decrease in laccase activity at neutral/alkaline pH may be due to the binding of a hydroxide anion (a laccase inhibitor) to the T2/T3 coppers of laccase and disrupt the internal electron transfer from T1 to T2/T3 centres thereby inhibiting the enzyme activity [48]. The effect of pH on laccase stability was studied to determine the optimum conditions required for enzyme purification, application, and storage. Laccase from *Trametes polyzona* WRF03 was more stable towards neutral pH (5.5–6.5) while the stability was relatively low at acidic pH (below 4.0). Chairin et al. [46] reported stability of laccase from *Trametes polyzona* WR710–1 between pH 6.0 and 7.0 whereas Sayyed et al. [49] reported higher stability of laccase at neutral pH. On the other hand, the stability of laccase at alkaline values was reported for laccase from *Perenniporia tephropora* at pH 8.0 [29], laccase from *Cerrena unicolor* at pH 9.0 [50]. Stability of the laccase from *Trametes polyzona* WRF03 was quite high over a broad pH range. This could be a very important characteristic for industrial applications.

The optimum temperature of 55 °C exhibited by *Tp*WRF03 laccase is the same as that reported for laccase from *Ganoderma lucidum* [51] and comparable with 50 °C for laccase from *Trametes polyzona* KU-RNW027 [44], and 60 °C *Shiraia* sp SUPER-H168 [52]. According to Baldrian [2], the temperature profiles of laccase just like other ligninolytic enzyme system has optimum temperature between 50 °C and 70 °C. In addition, the *Tp*WRF03 laccase was more stable at 40 and 50 °C over the time course of the heat treatment. Similarly, Lueangjaroenkit et al. [44] and Chairin et al. [46] reported stability up to 50 °C for 1 h for *Trametes polyzona* KU-RNW027 and *Trametes polyzona* WR710–1 laccase respectively. The ability of *Tp*WRF03 laccase to act on a broad temperature range of 25–100 °C and stability at 40 °C and 50 °C makes it a valuable biocatalyst for many biotechnological applications.

Organic solvents are important non-aqueous media commonly used for biocatalysis [52]. Hence, determination of laccase stability in various organic solvents will provide useful information for the choice of suitable reaction media for its application in biocatalysis and biotransformation. Resistance to water-miscible solvents is an important consideration for industrial application of laccase since many transformations are at high concentrations of organic solvents. Laccases generally show high sensitivity to unfolding in the presence of these compounds [53]. Laccase from *Trametes polyzona* WRF03 showed a relatively gradual decrease in stability with a 10 % increase in the concentration of various organic solvent. Lueangjaroenkit et al. [44] obtained similar result trend for laccase from *Trametes polyzona* KU-RNW027 except in the presence of 10–30 % butanol. The *Tp*L was more stable in acetone (50 %), methanol (45.65 %) and propanol (41.57 %) than in acetonitrile (31.18 %) and ethanol (14.55 %) when exposed to these solvents for 1 h. The result obtained in the present study showed higher resistance to inactivation in the presence of organic solvents unlike laccases from *Marasmius quercophilus* and *Chalara paradoxa* [54,55]. Although the enzyme is relatively stable at different organic solvents, its activity was lower when compared to the aqueous solution.

The effect of metal ions on enzyme activity is highly considered in industrial application since some metal ions have inhibitory as well as the stimulatory effect on the enzyme activity. This is imperative as metal ions are common environmental pollutants and can affect both the production and stability of the extracellular enzymes [56]. The result of this study showed that all the metal ions studied reduced *Tp*L activity differently except $Mg^{2+}$ and $Cu^{2+}$ that showed slight stimulatory effect on laccase activity. A similar observation was found in other fungi. Castano et al. [57] observed a slight stimulatory effect of copper ions ($Cu^{2+}$) on laccase from *Xylaria* sp. In addition, Forootanfar et al. [58] and Si et al. [59] reported similar observation for laccases from *Paraconiothyrium variabile* and *Trametes pubescens* respectively. The activation of laccase activity at higher $Cu^{2+}$ maybe due to the saturation of type 2 (T2) copper binding sites of the enzyme with $Cu^{2+}$ at this concentration [6]. Since white rot fungal laccases are multi-copper-containing oxidases, cupric ions show varied (positive, negative, or neutral) effects on laccase activities [22]. In contrast, Zhou et al. [60] reported inhibition of laccase from *Odontotermes formosanus* by $Cu^{2+}$. However, there was no effect of cupric ions on laccases from *Cerrena* sp. HYB07 [61], and *Clitocybe maxima* [41].

Furthermore, *Trametes polyzona* WRF03 laccase activity was inhibited by increasing the concentration (10–50 mM) of metal ions from metallic chlorides such as $Ca^{2+}$, $Co^{2+}$, $Hg^{2+}$, $Ba^{2+}$ and $Fe^{2+}$. This was consistent with the report by Zhao et al. [62] for laccase activity for *Myrothecium verrucaria* NF-05. $Fe^{2+}(FeSO_4)$ strongly inhibited the enzyme activity than any other metal ion tested. Similar results have been obtained for laccases from *Trametes polyzona* WR710-1 [46] and *Paraconiothyrium variabile* [58]. On the contrary, $Fe^{2+}$ did not inhibit laccase from *Trametes pubescens* [59]. Apart from metal ions inhibition, the inhibitory effect of several other putative inhibitors such as sodium cyanide, sodium azide, SDS, urea, L-cysteine and EDTA were tested on *Tp*L. All the inhibitors showed more than 50 % inhibition at 5 and 10 mM respectively although their percentage inhibition differs with the type/concentration of the inhibitors. Complete inhibition of laccase activity was observed with sodium azide and sodium cyanide. Similar result was obtained by Lueangjaroenkit et [44] with sodium azide. Sodium azide and Sodium cyanide are common inhibitors of metallo-proteins and have been reported as potent inhibitors of laccase-catalysed reactions [63].

The purified laccase from *Trametes polyzona* WRF03 showed the ability to decolourise a broad class of structurally different dyes although with different decolourisation efficiency. Several reports have highlighted the decolourisation of azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes by laccases [64,65]. The present study showed that laccase from *Trametes polyzona* WRF03 could decolourize azo-dyes such as methyl orange, eriochrome black and congo red. Laccase breakdown azo-dye structures by oxidizing their chromophoric assemblies with subsequent generation of free radicals [66].

Similarly, the present study also revealed that laccase from *Trametes polyzona* WRF03 was able to decolourize triphenylmethane and triarylmethane dyes such as coomassie brilliant blue and malachite green with greater percentage decolourisation efficiency of 58 and 72 %, respectively. These two classes of dyes were also found to be toxic and recalcitrant in nature [67]. This may be because the chemical structure of the dyes contains electron-donating methyl and methoxyl groups. The result was in agreement with results previously reported for *Trametes* sp SQ01 laccase [68], *Pleurotus ostreatus* [69], *Paraconiothyrium variable* laccase [58] and *Pycnoporus sanguineus* laccase [70]. The differences in their decolourisation rates may be due to the purification steps adopted, the specific catalytic efficiency of the enzyme, structural properties of the dyes and the concentration used. Furthermore, the laccase from *Trametes polyzona* WRF03 was not efficient in decolourising thiazin dye (Azure B) and heterocyclic dye (methylene blue). Similarly, there was no decolourisation (0%) of methylene blue by laccase from *Perenniporia tephropora* even in the presence or absence of HBT [29].

The lack of characteristic peak at 610 nm indicated that laccase from *Trametes polyzona* WRF03 is a yellow laccase. However, most of the laccase characterised so far are blue laccases. Yellow laccase varies from blue laccase by the loss of absorption band around 610 nm. The loss of the absorption band at 610 nm of the yellow laccase may due to the displacement of the cupric ions in the active centre of laccase responsible for its absorptive properties by another metal atom such as zinc and iron [71]. However, Leontievsky et al. [72] related this loss of spectral property due to the presence of the copper atoms in their reduced state. The existence of incomplete oxidation state of copper ($Cu^{+}$), with a fully occupied electron configuration of d10 resulted in no d-d transition and therefore the lack of absorption at 610 nm. Secondly, yellow laccase oxidizes non-phenolic substrates in the absence of mediator molecules [72], which are required in case of blue laccase. Thus, yellow laccase is a better biocatalyst than blue laccase [31].

## 5. Conclusion

The molecular screening strategy using laccase gene-specific primers showed that *Tp*WRF03 produces a true laccase. *Tp*WRF03 laccase (*Tp*L) relatively possesses striking enzymatic properties (stability over a broad pH and temperature ranges and organic solvent tolerance) that are relevant for application in myriads of industrial processes requiring oxidation-reduction reactions. In addition, the enzyme showed relatively high decolourisation efficiency towards different classes of textile dyes that are structural analogues of lignin and many recalcitrant environmental pollutants. Thus, the enzyme may find application in bioremediation especially in wastewater containing dyes and other aromatic pollutants.

## CRediT authorship contribution statement

**Tobechukwu Christian Ezike:** Investigation, Data curation, Formal analysis, Writing - original draft, Visualization. **Arinze Linus Ezugwu:** Writing - review & editing. **Jerry Okwudili Udeh:** Validation. **Sabinus Oscar Onyebuchi Eze:** Supervision. **Ferdinand Chiemeka Chilaka:** Conceptualization, Methodology, Supervision.

## Declaration of Competing Interest

None.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00566.

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