Characterization and purification of the crude *Trematosphaeria mangrovei* laccase enzyme

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Objectives
The aim of this work was to study the purification and characterization of the crude extracellular laccase produced by the marine-derived fungus *Trematosphaeria mangrovei.*

Methods
The general properties of the crude laccase enzyme produced by *T. mangrovei* were investigated. These include the effect of temperature, pH, thermal and pH stabilities, and enzyme and substrate concentrations on the laccase activity. Partial purification of the *T. mangrovei* laccase enzyme was carried out by fractional precipitation with ammonium sulphate, ethanol and acetone. Further purification was carried out on a Sephadex G-100 column.

Results and conclusion
The results obtained showed that the crude enzyme reached its maximal activity at 35°C, pH 4.5, at an enzyme concentration of 5.429 mg protein/reaction mixture and at a substrate concentration of 40 mmol/l 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid). The enzyme was stable for 60 min at 35°C and retained about 80–90% of its activity after treatment for 60 min from 40 to 50°C. The enzyme showed maximum stability (100%) at pH 4.5 and 91.6% at pH 4.0 after 60 min. Fractional precipitation of the fungal extracellular *T. mangrovei* laccase enzyme with different methods showed that the enzyme fraction precipitated at 60% acetone was the most favourable enzyme fraction; it showed 4.84 purification fold. Laccase obtained from the 50–60% acetone fraction was purified by Sephadex G-100. The final preparation thus obtained reached 31.47-fold that of the culture filtrate (1466.49 U/mg protein) and showed a single band on native polyacrylamide gel electrophoresis.

Keywords:
characterization, laccase, lignin-degrading enzymes, ligninolytic enzymes, marine-derived fungi, purification, *Trematosphaeria mangrovei*

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Introduction

Laccase (E.C.1.10.3.2, *p*-benzenediol : oxygen oxidoreductase) is a copper-protein belonging to a small group of enzymes denominated blue oxidase. It is an oxidoreductase that can catalyse the oxidation of various aromatic compounds (particularly phenols) with the concomitant reduction of oxygen to water [1]. Moreover, in the presence of primary substrates that act as electron transfer mediators, such as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 1-hydroxybenzotriazole, the substrate range can be extended to nonphenolic compounds [2]. Laccase or laccase-like activity has been found in higher plants, some insects and a few bacteria [3]. However, most known laccases are from fungi, especially from the white rot fungi. Finally, although most laccases have been characterized from white rot basidiomycetes, there are other groups of fungi (marine-derived fungi) that produce laccases but they have been studied to a much lesser extent [4].

Fungal laccases are involved in various processes in nature including the biodegradation of lignin [5] and their application in the detoxification of various aquatic and terrestrial pollutants and in the treatment of industrial wastewater has been suggested [6]. Laccase production by the filamentous marine-derived fungus *Trematosphaeria mangrovei* has been described in a previous study [7].

The characterization and purification of the crude laccase enzyme obtained from the optimized fermentation medium is described in this work.

Materials and methods

Chemicals

ABTS diammonium salt was obtained from MP Bio (LCN) (USA). Sephadex G-100 was purchased from Fluka Company (Germany). The standard of the laccase

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enzyme from *Trametes versicolor* was purchased from Sigma Chemical Company.

The fungal strain and culture condition

The filamentous marine-derived fungus *T. mangrovei* used in this study was isolated from decayed wood samples collected from Abou Keer (Alexandria, Egypt), and identified in the National Research Centre, Chemistry of Natural and Microbial Products Department (Microbial Culture Collection Unit) according to Kohlmeyer and Kohlmeyer [8]. The final optimized medium composition was found to be as follows (g/l): 16 sucrose, 2 peptone, 1 yeast extract, 50% sea water and addition of copper sulphate (2.5 mmol/l) on the sixth day of incubation with a 20-day incubation period. The optimum initial pH was 6 during incubation at a temperature of 25°C under static conditions.

Assay of laccase activity

Laccase (EC 1.10.3.2) activity was measured using the method described by Bourbonnais *et al.* [9] on the basis of the oxidation of the substrate ABTS. The rate of ABTS oxidation was determined spectrophotometrically at 420 nm.

The reaction mixture contained 600 µl sodium acetate buffer (0.1 M, pH 5.0 at 27°C), 300 µl ABTS (5 mmol/l), 300 µl culture filtrate and 1400 µl distilled water. The mixture was then incubated for 2 min at 30°C and the absorbance was measured immediately at 1-min intervals. One unit of laccase activity was defined as the activity of an enzyme that catalyses the conversion of 1 mol of ABTS per minute.

Determination of total proteins

The protein content of the culture filtrate was estimated according to the method of Lowry *et al.* [10].

General properties of the crude laccase enzyme

Effect of the temperature of the reaction

In the present experiment, identical reaction mixtures were incubated at different temperatures (15–60°C) for 2 min at pH 5.

Effect of the pH value of the reaction

This experiment was conducted to determine the optimum pH value at which the enzyme showed its maximal activity. The reaction mixtures of different pH values (3.5–7.0) were prepared using 0.1 mol/l acetate buffer (pH 3.5–5.5) and 0.1 mol/l phosphate buffer (pH 6.0–7.0).

Thermal stability of the crude laccase

The effect of temperature on enzyme stability was studied by preheating the crude enzyme in 0.1 mol/l acetate buffer (pH 4.5) at different temperatures (35–70°C) for different time intervals (15–60 min). The activity was then measured at 35°C. Controls were carried out using the enzyme solutions without preheating and its activity was taken as 100%.

pH stability of the crude laccase

In the present experiment, the crude enzyme solution was subjected to different pH values using acetate buffer pH 3.5–5.5 and phosphate buffer pH 6.0–7.0 at 0.1 mol/l for each buffer for different periods at 35°C. The residual activity was assayed after each incubation period.

Effect of enzyme concentration

The reaction was carried out under standard conditions with varying amounts of crude enzyme (0.181–9.047 mg protein/reaction mixture), and then the enzyme activity was determined.

Effect of substrate concentration

The effect of substrate concentration on the crude enzyme activity was studied using different concentrations of ABTS ranging from 1 to 100 mmol/l in the reaction mixture. The reaction was carried out at pH 4.5 at 35°C and incubated for 2 min. Controls were prepared using the same substrate concentrations and dead enzyme.

Partial purification of laccase enzyme

Fractional precipitation of the enzyme preparation was achieved with ammonium sulphate, ethanol or acetone. In all cases, an ice-salt bath was used and the precipitant was added to the cold culture filtrate until the required concentration was achieved. After isolating the precipitated fraction by centrifugation in a refrigerated centrifuge, the supernatant was subjected to further precipitation and the process was repeated. Each fraction was suspended in distilled water and dialysed against distilled water in a refrigerator [11].

Purification of the laccase enzyme

The enzyme fractions obtained from acetone 60% concentration were collected, lyophilized and applied to a column (65 × 2.0 cm) packed with 50 ml volume of Sephadex G-100 [11].

The column was eluted with 0.1 mol/l acetate buffer (pH 5.0). Five milliliter fractions were collected at a flow rate of 15 ml/h. The protein content and laccase activity were determined.

Native polyacrylamide gel electrophoresis

It was carried out according to Smith [12].

Results

General properties of the crude laccase enzyme preparation from *Trematosphaeria mangrovei*

In this series of experiments, the general properties of the crude laccase enzyme produced by *T. mangrovei* grown on the optimized culture medium were determined; these included the effects of temperature and pH of the reaction mixture, thermal and pH stability as well as the effects of enzyme and substrate concentrations on laccase activity.

Effect of temperature of the reaction mixture

Data presented in Fig. 1 show that the enzyme reached its maximal activity at 35°C, followed by 40°C; deviation of temperatures beyond this range had adverse effects on enzyme activity. At 35 and 40°C, the laccase activity increased 11.38 and 3.29%, respectively, compared with the control 100% at 30°C.

Effect of the pH value of the reaction mixture

From Fig. 2, it can be seen that the activity of the enzyme was increased in the acidic range until the maximum was achieved at pH 4.5, and then decreased markedly with increasing ionic strength of the buffer to alkaline. At pH 4.5, the laccase activity increased 62% as compared with the control 100% at pH 5.0; higher alkalinity led to a decrease in the activity until the enzyme became inactive at pH 6.5–7.0.

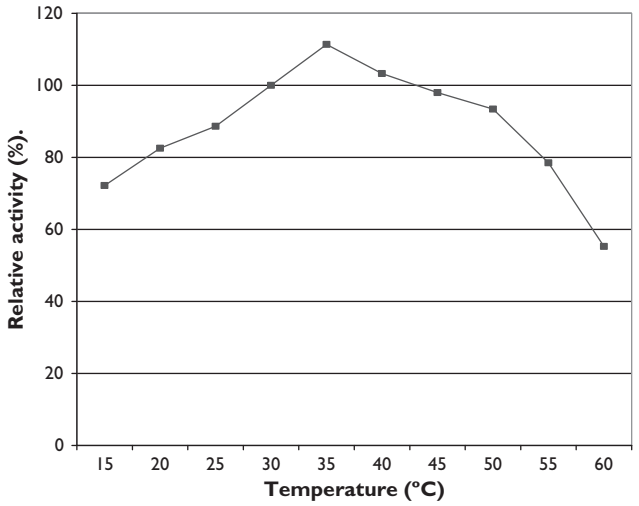
Thermal stability of the crude laccase

The results presented in Fig. 3 show that the stability of the enzyme activity depended on the temperature and the time of heating. At 35°C, the enzyme was stable for 60 min However, the enzyme retained about 80–90% of its activity after treatment for 60 min at 40°C up to 50°C. At 55 and 60°C, the enzyme began to lose its activity partially. More adverse effect was observed on heating at 65 and 70°C, with a loss of ~77.89 and 84.31%, respectively.

pH stability of the crude laccase

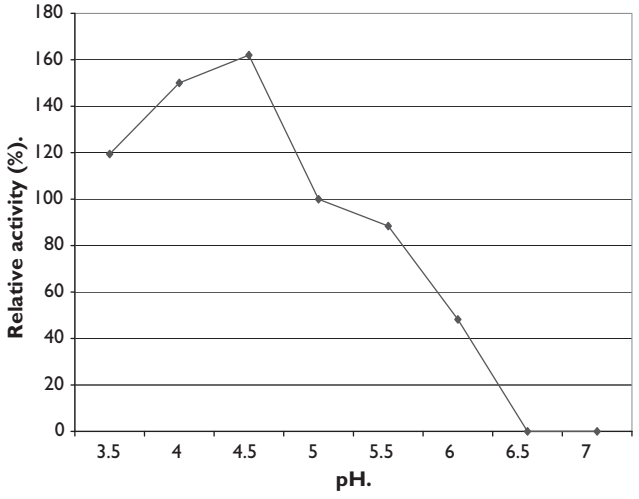
The results presented in Fig. 4 show that at pH 4.5, the enzyme activity had the highest stability after 60 min of exposure. Furthermore, at pH 4, the enzyme retained about 91.60% of its activity after 60 min of exposure.

Figure 1



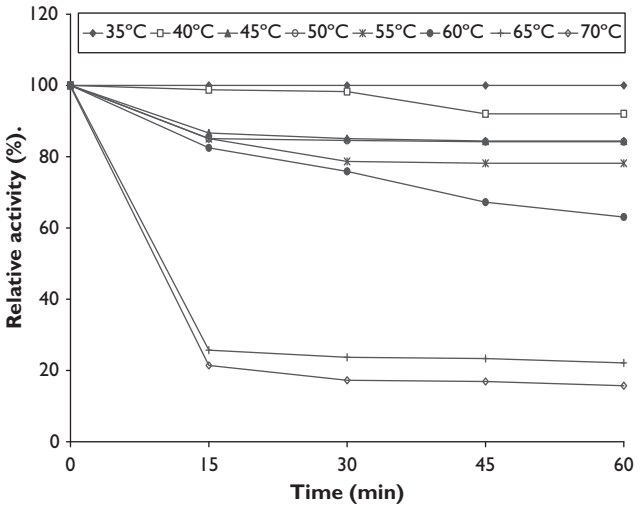
Effect of temperature of the reaction mixture on crude laccase enzyme activity.

Figure 2



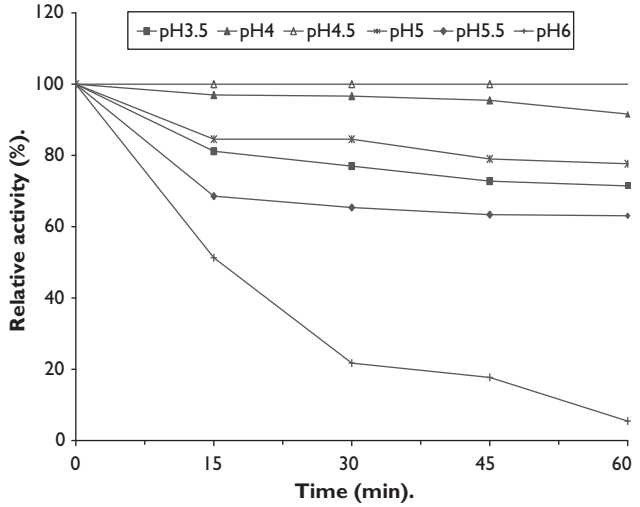
Effect of pH of the reaction mixture on crude laccase enzyme activity.

Figure 3



Thermal stability of the crude laccase enzyme from *Trematosphaeria mangrovei* at pH 4.5.

Figure 4



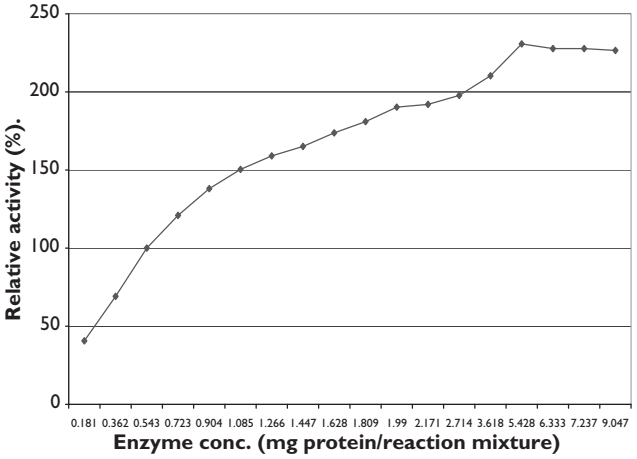
pH stability of the crude laccase enzyme from *Trematosphaeria mangrovei* at 35°C.

At pH 3.5 and 5.0, the enzyme lost about 28.55 and 22.33% of its activity after 60 min, respectively. The results also indicated that pH 5.5 had an adverse effect on the enzyme activity; about 36.94% of the enzyme activity was lost after 60 min, whereas it lost 94.54% of its activity after 60 min of exposure at pH 6.

Effect of enzyme concentration on crude enzyme activity

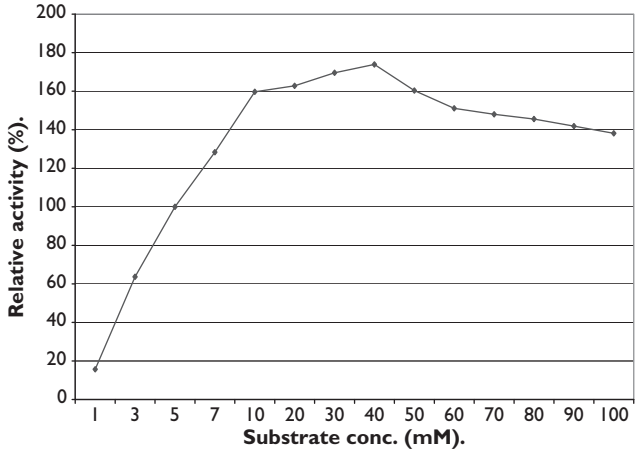
The results presented in Fig. 5 show that the enzyme concentration had a major effect on the enzyme activity. The activity of enzyme was increased directly with protein increase. The maximum of enzyme activity was obtained by the enzyme concentration from 0.181 to 5.429 mg per reaction mixture. At the enzyme concentration of 5.429 mg protein, the laccase activity increased 130.74% compared with the control experiment, which was 100% at 0.543 mg protein. However, on further increasing the enzyme concentration, the activity remained almost constant.

Figure 5



Effect of different enzyme concentrations on crude enzyme activity.

Figure 6



Effect of substrate concentrations on crude enzyme activity.

Effect of substrate concentration on crude enzyme activity

The data presented in Fig. 6 indicate that the substrate concentration 40 mmol/l ABTS was the optimum for the laccase enzyme; the enzyme activity increased 73.85% as compared with the control. The increase in enzyme activity was parallel to the increase in the substrate concentration until it reached a maximum at 40 mmol/l ABTS.

Fractional precipitation and partial purification *of* the *Trematosphaeria mangrovei* laccase enzyme

Partial purification of the *T. mangrovei* laccase enzyme was carried out by fractional precipitation with ammonium sulphate, ethanol and acetone. A total of 21 fractions were obtained, including seven with ammonium sulphate, seven with ethanol and seven with acetone.

The laccase activities recovered by precipitations were 0.34, 2.75 and 2.82%, respectively. Of all the fractions obtained by three precipitants, the fraction obtained by precipitation at the 50–60% acetone concentration showed the highest specific laccase activity (225.68 U/mg protein) and good enzyme activity (8734.79 U). In addition, the highest specific activity (225.68 U/mg protein) of this fraction reached 4.84-fold of the culture filtrate; therefore, this fraction (38.71 mg protein) was further purified by Sephadex G-100 column chromatography.

Gel filtration on a Sephadex G-100 column

Laccase enzyme fractions obtained by 50–60% concentration of acetone were collected, lyophilized and subjected to further purification on Sephadex G-100 (Fluka Company) column. Elution was performed with 0.1 mol/l acetate buffer (pH 5.0). The results are presented graphically in Fig. 7.

The column yielded two protein components: the first was the minor component (8.86 mg protein) covered by fractions 6–10 and had the highest recovered activity (8440.443 U). The second was the major component (26.79 mg protein) covered by fractions 11–20 and had weak enzyme activity, indicating that this protein component was not related to laccase.

The activity of laccase enzyme covered by fractions 6–14 represented about 96.91% of the applied activity. The fraction number 8 of the laccase component was the most active and showed 6.5-fold purification.

The purification scheme of the *T. mangrovei* laccase enzyme showed that the fraction number 8 had the highest specific activity (1466.49 U/mg protein) and the highest purification, 31.47-fold, compared with the culture filtrate (Table 1).

The purity of laccase enzyme fraction 8 was determined by native polyacrylamide gel electrophoresis.

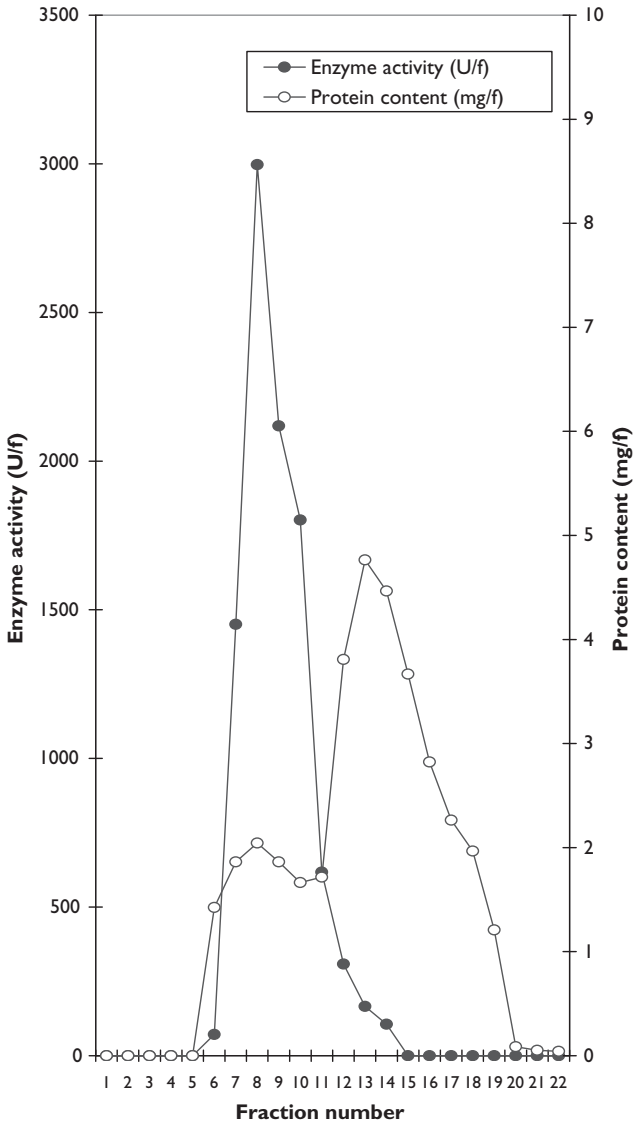
Discussion

After optimization of the chemical composition of the production medium, the general properties of the crude

laccase enzyme were determined. These included the effects of temperature and pH of the reaction, thermal and pH stability and also the effects of enzyme and substrate concentrations.

The enzyme showed an optimum temperature of 35°C and it was stable at 40°C up to 50°C, at which the enzyme retained about 90–80% of its activity. It retained about 78.14% of its initial activity after 60 min of incubation at 55°C. The enzyme began to lose large amounts of its activity on heating at 65 and 70°C. This result is similar to that reported by Sadhasivam *et al.* [11], who found that the *Trichoderma harzianum* WL1 laccase enzyme was active in a temperature range from 30 to 50°C, with the maximum activity at 35°C, and the *T. harzianum* laccase enzyme retained 70% of its initial activity after 1 h of incubation at 55°C. However, Cambria *et al.* [13] found that the laccase from *Rigidoporus lignosus* had the maximum activity at 40°C.

Figure 7



Gel filtration on a Sephadex G-100 column.

The crude enzyme showed its maximum activity at pH 4.5 in 0.1 mol/l sodium acetate buffer, which was similar to the result of Sadhasivam *et al.* [11], who reported that the maximum laccase activity of *T. harzianum* WL1 was observed at 4.5 pH when ABTS was used as a substrate, and at pH values higher than 4.5, the enzyme activity decreased gradually and was completely inactivated at higher alkaline pH. This phenomenon can be attributed to the difference in the redox potential between a reducing substrate and the type 1 copper in the active site of the enzyme and the inhibition of type 3 copper by hydroxide ion at a higher pH [14].

On studying the pH stability of the *T. mangrove*i laccase enzyme at 35°C, the results showed that the enzyme was more stable at pH values of 4.0 and 4.5 [15]. It was found that the *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH32 laccase enzyme was stable in a pH range from 4.0 to 9.0, but it was inactive at pH 3.0.

The results indicated that the optimum enzyme and substrate concentrations in the reaction mixture were 5.429 mg protein and 40 mmol/l ABTS, respectively.

Partial purification of the fungal extracellular laccase enzyme was achieved by fractional precipitation with ammonium sulphate, acetone and ethanol.

From the 21 fractions obtained, the enzyme fraction precipitated at 60% acetone was the most active (2.82% recovered activity, 225.68 specific activity) and had the highest purification: 4.84-fold. Therefore, it was the most favourable enzyme fraction and was used in future work. These results are higher than those reported by Sadhasivam *et al.* [11] for the *T. harzianum* WL1 laccase enzyme (1.30 specific activity and 1.51-fold purification).

Further purification of the partially purified extracellular laccase enzyme (the fraction precipitated at 60% acetone concentration) was achieved when it was loaded onto a Sephadex G-100 column.

When the laccase enzyme was covered by fractions 6–14, its activity represented about 96.91% of the applied activity. The fraction number 8 of the laccase component was the most active and showed 6.5-fold purification compared with the loaded sample (60% acetone) and 31.47-fold purification compared with the culture filtrate.

Table 1 Purification scheme of the *Trematosphaeria mangrovei* laccase enzyme

Steps of purification	Total activity (U/fraction)	Protein content (mg/fraction)	Specific activity (U/mg/fraction)	Yield (%)	Purification fold
Culture filtrate (crude enzyme)	309 814	6648.70	46.60	100	1
Precipitation with 60% acetone (partial purified enzyme)	9945.23	44.09	225.58	3.21	4.84
Sephadex G-100 column chromatography	2997.49	2.04	1466.49	0.97	31.47

This result is similar to that reported by Sadhasivam *et al*. [11] for the *T. harzianum* WL1 laccase enzyme (30.6-fold purification).

Conclusion

Fungal laccases are involved in various processes in nature including the biodegradation of lignin, detoxification of various aquatic and terrestrial pollutants and treatment of industrial wastewater. The crude enzyme reached its maximal activity at 35°C, pH 4.5, at an enzyme concentration of 5.429 mg protein/reaction mixture and at a substrate concentration of 40 mmol/l ABTS. The enzyme was stable for 60 min at 35°C and retained about 80–90% of its activity after treatment for 60 min from 40 to 50°C. The enzyme showed maximum stability of 100% at pH 4.5 and 91.6% at pH 4.0 after 60 min. Fractional precipitation of the fungal extracellular *T. mangrovei* laccase enzyme by different methods showed that the enzyme fraction precipitated at 60% acetone was the most favourable enzyme fraction, showing 4.84-fold purification. Laccase obtained from the 50–60% acetone fraction was purified by Sephadex G-100. The final preparation thus obtained reached 31.47-fold that of the culture filtrate (1466.49 U/mg protein) and showed a single band on native polyacrylamide gel electrophoresis. Thus, *T. mangrovei* is an excellent producer of laccase, especially for use in biotechnological processes.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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