PURIFICATION AND CHARACTERIZATION OF LACCASE INVOLVED IN THE DECOLOURIZATION OF SYNTHETIC DYES AND 2,3,7,8-TCDD CONGENER DEGRADATION BY THE WHITE ROT FUNGUS ISOLATED FROM BAVI FOREST OF VIETNAM

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ABSTRACT

The fungal strain FBV40 was isolated from soil containing decayed wood in Ba Vi National Forest and capable of producing an extracellular laccase in the TSH1 medium. Two isozyme such as Lac1 and Lac2 were purified were estimated to be 55 and 60 kDa by SDS-PAGE. The optimum pH and temperature for the enzymatic activity of Lac1 were 3.0 and 60°C with ABTS using as the substrate. Kinetic constants K_m and V_{max} of Lac 1 were 0.3 µM and 200,000 µM/mins with ABTS as substrate. Cl^-, SDS, and EDTA at any concentration (2 mM; 5 mM and 10 mM) strongly inhibited the activity of laccase. The enzyme was stable in the presence of several metal ions including Ni^{2+} (1 mM), Cu^{2+} (1 mM and 3 mM), Ca^{2+} (3 mM and 4 mM); in the presence of Cu^{2+} (2 mM) and Ca^{2+} (0.5 mM, 1.0 mM and 2.0 mM), laccase even showed the increase in the activity. The presence of metal ions Mn^{2+}, Mg^{2+}, Fe^{2+} completely inhibited the enzymatic activity at any examined concentration. The crude enzyme, as well as Lac 1, was able to decolourization MN.FBN dye from the textile industry from Ministry of Defence. This strain was able to degrade 2,3,7,8-TCDD isotop with initial concentration 2,000 ng-TEQ/L at rates over 46.8 % after ten days cultivation in the TSH1 medium. In the presence of three strains FBV40, FBVLa1 and FBD154 with the ratio 1:1:1, the degradation of this congener was achieved more than 95 % at the same time cultivation.

Keywords: laccase, 2,3,7,8-TCDD, Ba vi forest, degradation, decolourization.

1. INTRODUCTION

Waste water from textile industries is a complex mixture of many polluting substances including organochlorine-based pesticides, heavy metals, pigments and dyes, which causes a lot of difficulties for the degradation process. To counter these issues, white rot fungi with flexible

enzymes that capable of degrading a wide board of aromatic compounds have been investigated for their potential application in textile effluent treatment.

Polychlorinated dibenzo–p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are part of the larger family of polyhalogenated aromatic hydrocarbons, which are well-known environmental pollutants. These pollutants are produced unintentionally by human activities. Studies of the degradation of PCDDs and PCDFs in the environment have known these rates to be extremely low [1]. There have been two reports of the deterioration of 2,3,7,8-TCDD and even more highly chlorinated DDs and DFs by *Phanerochaete chrysspodium* and *Phanerochaete sordida*. Although the degradation of tetra- to octa- CDDs and CDFs by these fungi was observed, the metabolic mechanism was not elucidated. In this study, we describe the purification and characterization of the laccase from fungus from Ba Vi forest of Vietnam, as well as the first data about of white rot fungus that can degrade 2,3,7,8-TCDD, the most toxic form of dioxin.

2. MATERIALS AND METHODS

2.1. Fungal strains and culture conditions

The fungal strain FBV40 was isolated from soil containing decayed wood in Ba Vi National forest, Vietnam. The strains FNBLa1 and FBD154 were obtained from the Environmental Bioremediation Laboratory (EBR).

2.2. Laccase enzyme assay

Extracellular laccase activity was measured spectrophotometrically with ABTS.

2.3. Protein Quantitation

The protein concentration of the crude and purified enzyme was determined by the Bradford method using bovine serum albumin (BSA) as standard [2].

2.4. Purification of laccase

Crude enzyme obtained from FBV40 was centrifuged at 8,000 × g for 15 minutes and then concentrated by ultrafiltration with a PM-10 membrane (10 kDa). The concentrated enzyme was applied to a Sephadex G-75 (2 × 25 cm glass) column pre–equilibrated with sodium acetate buffer (20 mM, pH 5.5). Proteins were eluted with the same buffer and the flow rate maintained at 0.5 mL min^{-1}. Fractions of 1 ml were collected, and those with high enzyme activity were pooled and subjected to the anion exchange column on Hitrap Q-HP (1 cm × 5 cm), equilibrated with the same buffer. Proteins were eluted with a linear gradient of NaCl (0–0.5 M) at a flow rate of 1.5 mL min^{-1}. The purification was carried out at the room temperature.

2.5. Characterization of purified laccase

The pH and temperature optima for the purified laccase were determined as follows. Purified laccase enzyme activity was determined by varying the pH 1.0–8.0 or the temperature (35–70 °C) of the reaction under standard assay conditions.

The effects of metal ions including Ni^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} and Co^{2+} (0.5–5 mM) and inhibitors including Cl-, EDTA, SDS and L-casein (2.5 and 10 mM) on laccase activity were investigated by incorporating them into assay mixture before determination.

Effect of purified laccase on different substrates including ABTS, 2,6-DMP, guaiacol, and syringaldazine was also investigated. Michaelis–Menten kinetics were used to determine K_m and V_{max} using ABTS as the substrate (0.1- 0.5 mM) and the kinetic constants (K_m and V_{max}) were derived from Lineweaver-Burk plots.

2.6. Decolorization of dye

The reaction mixture of the decolorization experiment contained 50 mg/L dye, crude or Lac1 (final concentration, 1000 U/L), Vio at different concentrations (0, 50, 100, 200, 300, 400 and 500 μM) and 20 mM sodium acetate buffer (pH 3).

2.7. 2,3,7,8-TCDD degradation

The reaction mixture contained 15 ml of culture supernatant of strain FBV40 or the mix strains consist of FBV40, FBD154 and FNBLa1 (1:1:1) and purity 2,3,7,8-TCDD (supplied by BE-Basic Company-The Netherland) solubilized in DMSO with the final concentration of 2,000 ppt-TEQ/L. The mixture was cultured in the TSH1 medium for 15 days. The whole culture was then homogenised and extracted with toluene (three times). The extracted solution was evaporated to 1 ml, and the remain of 2,3,7,8-TCDD concentration was determined using the gas chromatography- mass spectrometry (GC-MS).

3. RESULTS AND DISCUSSIONS

3.1. Purification of extracellular laccase

The extracellular laccase was purified to 4.7-fold with the yield of 98.8% after ultrafiltration 10 kDa. After gel filtration, the enzyme was purified up to 5.6-fold with the specific activity of 218 U/mg and a yield of 40.8 %. The laccase activity and protein concentration of each fraction after gel filtration column and anion exchange column were determined. The peak fraction was applied on Hitrap QHP column and eluted using NaCl 0.15 M. The collected enzyme was purified to 4.6-fold with a yield of 32.3 %. The final specific activity of the purified enzyme was 1016 U/mg protein.

Two isozymes were obtained after the purification step with the molecular mass of 55 kDa and 60 kDa when compared to authentic standards on SDS-PAGE and were called Lac1 and Lac2 respectively. The isozyme with the molecular mass of 55 kDa showed the dominant with a strong band when compared to the other and was chose for futher characteristic study.

3.2. Characterization of purified enzyme

3.2.1. Effects of pH on laccase activity and pH stability

Laccase exhibited the highest activity at pH 3.0. Enzyme activity decreased sharply as the pH value increased from 3.0 towards 6.0, and was completely inactive at pH 1.0. Laccase enzyme activity was stable at pH 5.0, retaining more than 50 % activity after 5 hours.

3.2.2. Effect of temperature on activity and stability of the purified laccase

The optimum temperature of Lac1 was determined to be 60 °C. The Lac 1 of FBV40 is a thermostable enzyme.

3.2.3. Effect on different substrates

The purified laccase from FBV40 was able to oxidize the typical laccase substrates including ABTS, syringaldazine (Syrin), 2, 6-DMP, and guaiacol (Gua). The purified enzyme exhibited the highest activity with ABTS.

3.2.4. Kinetic Parameters

The K_m value of the purified laccase was 0.3 µM, and its corresponding V_{max} value was 200,000 µM/min using ABTS as the substrate.

3.2.5. Effects of metal ions and inhibitors on the purified laccase

The purified enzyme was strongly inhibited by 10 mM of EDTA and Cl- (residual activity 0 %). The enzyme was not strongly inhibited by Cu^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} at the concentration 0.5 mM. Ca^{2+} increased laccase activity up to 107 % and 128 % at the concentration of 0.5 and 2 mM, respectively while Cu^{2+} increased laccase activity up to 109 % at 2 mM. Strong inhibition was caused by Fe^{2+} at any concentrations.

3.3. Decolorization efficiency of crude and purified laccase from FBV40

The highest decolourization of MN.FBN dye of crude and Lac1 after 24 h and at a concentration of ViO 500 µM were 92.67 % and 91.14 %, respectively. ViO had played important role in the decolourization of MN.FBN by laccase from strain FBV40.

3.4. Degradation of 2,3,7,8-TCDD

This strain directly degraded 2,3,7,8-TCDD purity congener with initial concentration 2000 ng TEQ/L at rates over 46 % after 10 days cultivation. In the presence of three strains FBV40, FNBLa1 and FBD154, the degradation of this congener was achieved more than 95 % at the same time cultivation (Fig. 1).

4. DISCUSSION

The molecular weight of FBV40 Lac1 was 55 kDa, it was similar to laccases from *Fomitiporia Mediterranea*n, *Trametes gallica* Lac 1, *Marasmius quero hilus*, *Polyporus* sp. [3]. Previous studies reported the molecular weight of 55 kDa of *Cerrena unicolor* CFC-120 and *Lentinus terminus* [4].The optimum temperature of laccase from FBV40 was similar to laccases of *Cerrena unicolor* MTCC 5159, *Clitocybe maxima, L. tigrinus*, and *Ganoderma lucidum* [4] and higher than the laccases from *Tricholoma mongolicum* (30 °C), *Pleurotus ostreatus* (35 °C) The result of the optimum pH 3.0 was accordance with other fungal laccases such as *Polyporus* sp., *Lentinus tigrinus, Tricholoma giganteum* [5]. Approximately 20 % and 35 % of Lac1 from FBV40 were remained after 60 minutes at 35 °C and 40 °C, respectively. Laccase activity from

Trametes Versicolor CCT 4521 was stable at 60 °C after 20 minutes incubation [5]. In the study of Ding *et al*. (2012), laccase of *G. lucidum* had the optimum temperature at 60 °C and remained 46 % activity at 60 °C after 80 minutes [6].

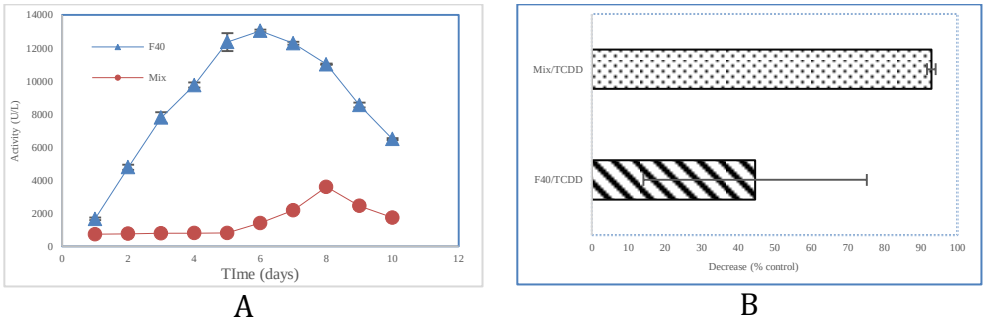

Figure 1. A) Time course of generation of laccase, and B) The degradation of 2,3,7,8-TCDD by single train and mix trains.

The purified laccase from FBV40 exhibited the highest activity toward ABTS, similar to laccases from other fungi, such as *C. maxima* [7], *Fusarium solani* and *Agrocybe cylindracea* [8]. Using ABTS as the substrate, the enzyme from *Trametes Versicolor* IBL-04 showed V_{max} of 780 U/mL with a corresponding K_m value of 73 µM [9]. The K_m value for the laccase purified from *T. hirsuta* showed more affinity to ABTS than 2,6-DMP [10].

White-rot fungi are capable of degrading a variety of recalcitrant aromatic pollutants, such as polyaromatic hydrocarbons (PAHs), polychlorinated phenols, and PCDDs. The degradation of these recalcitrants by white rot fungi is well correlated with their ligninolytic activities. The degradation of 2,7-diCDD could be achieved by the white-rot fungus *P. Chrys spodium* with the mechanisms included a LiP-catalyzed initial oxidation.

In this study, the degradation of 2,3,7,8-TCDD by the single white-rot fungus FBV40 and mix three strains were found. From the results of mass fragment patterns, it was predicted that the degradation of dioxin depends on the number of strains used to examine. To the best of our knowledge, this is the first report that the most toxic isotop of dioxin 2,3,7,8-TCDD could be degraded by laccase-producing fungi.

5. CONCLUSION

The molecular weight of the purified laccase of strain FBV40 were 55 kDa and 60 kDa by SDS-PAGE. The optimum and stable pH for the enzymatic activity was 3.0 with ABTS. Kinetic constants K_m and V_{max} of Lac1 were 0.3 µM and 200,000 µM/mins. The decolourization efficiency of crude and Lac 1 are similar. This strain directly degraded 2,3,7,8-TCDD congener with initial toxicity 2,000 ng TEQ/ml at rates over 46.7 % after 10 day cultivation. Using mixture of three strains FBV40, FNBLa1 and FBD154, the degradation efficiency of 2,3,7,8-TCDD was 92.9 % at the same cultivated condition.

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