Production and purification of laccase from *Trametes versicolor*

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Abstract – This study describes the production and purification of laccase from *Trametes versicolor* isolate. The activated fungus was grown in 50 mL YpSs broth medium for 3 days at 30 °C and 120 rpm shaking speed. Then, pellets were transferred to the laccase production medium including 5 g/L glucose, 1 g/L yeast extract, 1 g/L beef extract, 0.2 g/L pepton, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.1 g/L $CuSO_4$, 3 g/L $(NH_4)_2SO_4$, 1 g/L $CaCl_2$, and 1 g/L Na_2HPO_4. The highest enzyme activity was found after the sixth day of cell growth. For purification of laccase from crude extract, three-phase partitioning was used for the first time. The enzyme was purified by 9.5-fold with an enzyme activity recovery of 93% from the TPP system prepared at pH 7.0 with a ratio of 1:1 crude extract:t-butanol and containing 30% (w/w) ammonium sulphate. As a result, it is shown that the laccase enzyme from *T. versicolor* can be purified using three-phase systems, which are timesaving, inexpensive, and easy to use, instead of the traditional chromatography method.

Keywords –Trametes Versicolor, Laccase, Medium, Purification, Three-Phase Partitioning

I. INTRODUCTION

Laccases (E.C.1.10.3.2) are used as industrial enzymes in many applications, such as animal breeding, food industry, paper industry, bioremediation and biosensor production [1]. The enzyme laccase is a dimeric or tetrameric glycoprotein. The 3 redox sites contain four copper atoms in bound monomeric and multimeric forms; these copper atoms are prosthetic groups with high elongation within the group [2]. The copper of tip 1 is the structure that can be observed at 600 nm and gives the laccase enzyme a blue color. The type 2 copper zone is colorless. The visible region can be determined by EPR (electron spin resonance) despite its very weak absorption. The type 3 copper zone, on the other hand, contains a binuclear structure with double copper atoms; it shows no signals in EPR when listening to the attenuated absorption band at 330 nm [3-4]. In addition to escaping hydroxyl requirements of methoxy-linked monophenols, mid/para/diphenols, and polyphenols, laccases are also responsible for oxidation assemblies of aromatic amine, aniline, non-phenolic structures, and some inorganic ions in radical structures [5-6]. In addition, laccases can propagate dicarboxylic acids as well as phenolic and methoxyphenolic acids by attacking the methoxy structure [7].

In addition to creating a suitable environment for TPP (three-phase partitioning) proteins to remain in the liquid phase without degradation, it offers the advantages of short processing time, purification of the enzyme with high recovery, and low cost. The system consists of ammonium sulphate (salt) and t-butanol (organic solvent) attached to the crude enzyme extract. The components consist of two separate liquid phases (the upper alcohol-containing phase and the consuming salt-containing aqueous

phase). Ammonium sulphate is a salt that allows gradual "salting out" of the proteins. Butanol provides selective removal of pregnancy (low group weight lipids and phenolic substances) from biomolecules. Inexpensive centrifugation causes the proteins to pass into the pellet (interphase). Redissolving the interphase in buffer restores the specific and total activity, sometimes even increasing it. The most important advantage of TPP is that all regulations, large or small, can be applied [8].

Trametes versicolor, one of the Basidiomycetes species, is the best studied fungus producing laccase. Here we report the production and purification of laccase enzyme from *T. versicolor* using TPP.

II. Materials and Method

A. Fungal Growth, Reproduction and Storage

T. versicolor was kindly provided by Prof. Dr. Mustafa Yamaç (Biology Department, Eskişehir Osmangazi University). To obtain the mycelium of *T. versicolor*, growth was performed on PDA (Potato dextrose agar, Merck) solid medium. The sample taken from the stock culture was transferred to the centre of the Petri dishes containing PDA. It was then incubated at 30 °C for 10 days. The resulting mycelium was stored at +4 °C [9]. For growth in liquid culture, the mycelium was collected on day 3 with loops from the sample grown in PDA medium. The micelles were dissolved in 2 mL of distilled water in a sterile falcon by vortexing.

50 mL of the YpSs [10] broth medium was placed in a 250-mL flask, and the thawed mycelial suspension was added. The medium was incubated at 30 °C and 120 rpm for 3 days, and a preculture was established. After 3 days, the growing micelles were transferred to laccase production medium including 5 g/L glucose, 1 g/L yeast extract, 1 g/L beef extract, 0.2 g/L pepton, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.1 g/L $CuSO_4$, 3 g/L $(NH_4)_2SO_4$, 1 g/L $CaCl_2$, and 1 g/L Na_2HPO_4. The main culture was obtained by incubation at 30 °C and 120 rpm for 10 days [9-11].

B. Optimal Production of Laccase Enzyme

Growth in PDA medium was performed to qualitatively test for the presence of *T. versicolor* laccase. Guaiacol (0.02%, w/v), one of the laccase enzyme substrates, was added to the medium during growth and incubated at 30 °C for 3 days [12].

To achieve optimal production of *T. versicolor* laccase, growth was carried out for 8-10 days. From the first day, 1 mL sample was taken from the main culture every day and the activity was measured.

C. Determination of Laccase Activity

Laccase activity was determined by spectrophotometric method, using guaiacol as substrate. 5 mL of the reaction mixture containing 3.9 mL of acetate buffer (10 mM, pH 5.0), 1 mL of guaiacol (1.76 mM), and 0.1 mL of culture supernatant was incubated at 25 °C for half an hour. The absorbance value was then measured at 450 nm [13]. One unit of activity is the amount of enzyme required to oxidize 1 µmol of guaiacol per minute and was calculated according to the following formula [14].

$$Enzyme\ activity\ \left(\frac{U}{mL}\right) = \frac{\Delta OD}{\Delta t} x \frac{1}{\varepsilon} x \frac{Vt}{Ve} x\ DF\ x \frac{1}{incubation\ time\ (\text{min})}$$

ΔOD/Δt: change in absorbance versus time at 450 nm

ε: Guaiacol [12,100 M^{-1} cm^{-1}] molar absorption coefficient [15].

Ve: The volume of the enzyme sample in the assay cuvette

Vt: Total volume of the assay cuvette

DF: Dilution factor

D. Protein Determination

Protein concentration was determined by the Bradford method. Bovine serum albumin was used as protein to construct the standard curve [16].

E. Three-Phase Partitioning

In the study, 2 ml of crude enzyme extract was brought to different ammonium sulphate saturations (20%, 30%, 40%, 50%, 60%, and 70%, w/w) and a different ratio of crude enzyme extract to butanol (1.0: 1.0) was added to obtain t-butanol. The pH of the system was adjusted to 7.0. After the mixture was kept at room temperature for 1 h, it was centrifuged at 4500 rpm for 10 min and phase

separations were observed [17-18]. After the butanol-rich upper phase, in which no protein is expected, was removed by careful pipetting, the middle and lower phases were collected and dialyzed overnight against 100 mM sodium phosphate buffer solution (pH 7.0). The activity and protein levels in the middle and lower phases after dialysis were determined spectrophotometrically.

III. RESULTS

F. Qualitative Determination of Laccase Activity

Isolates of *T. versicolor* (30 °C) were seeded on PDA plates containing 5 mM guaiacol. Petri plates were incubated for 3-4 days. The presence of brown-coloured halos around the isolates, which oxidized guaiacol after incubation, was found to be positive for laccase enzyme production [19-20]. As shown in Figure 1, the production of extracellular laccase was observed in the *T. versicolor* isolate.

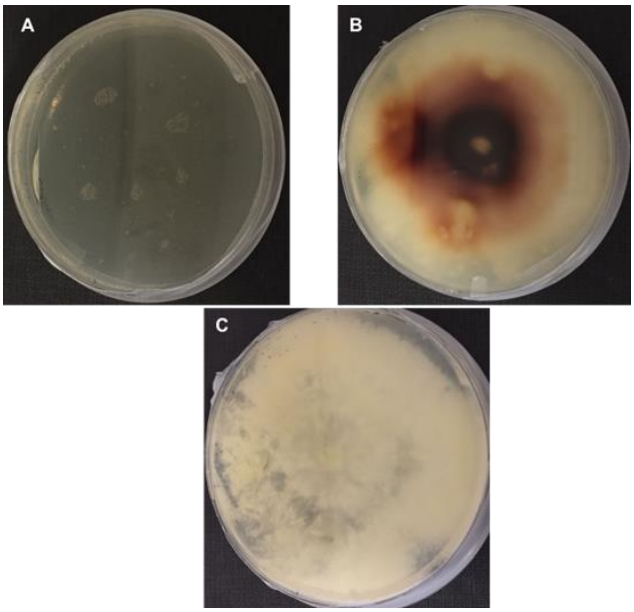

Figure 1. Qualitative determination of laccase in medium containing guaiacol (5 mM). A: dH_2O, B: *T. versicolor*, C. laccase negative strain (*Scytalidium thermophilum*)

G. Time Course of Laccase Production

For optimal production of laccase enzyme, the highest value of laccase activity was obtained on day 6 by growing for 8 days and determining laccase activity each day. The value of laccase activity, which increased regularly until day 6, peaked on day 6 and decreased rapidly after day 7 (Figure 2).

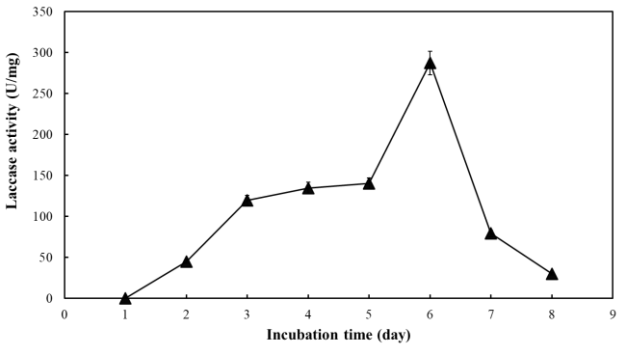

Figure 2. Time-dependent representation of the laccase activity of *T. versicolor* (U/mg)

H. Laccase Purification

The laccase of *T. versicolor* was purified by testing 20-70% ammonium sulphate salt concentrations with a 1:1 ratio of crude extract: t-butanol. In these experiments, the laccase enzyme was purified 9.5-fold, with a recovery of 93% at an ammonium sulphate concentration of 30% (Figure 3). Although the recovery was at a good level with increasing salt concentration, the purification coefficient decreased significantly.

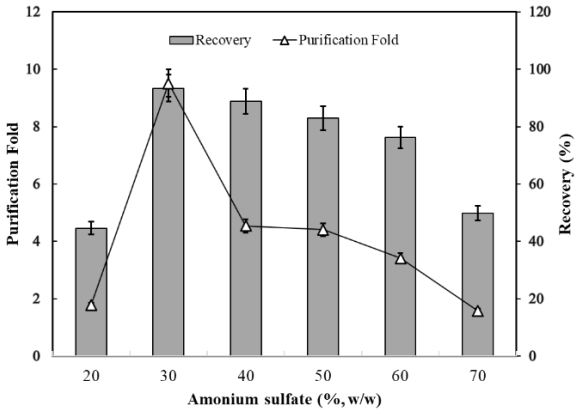

Figure 3. Ammonium sulphate concentration compared to three-phase separation.

IV. DISCUSSION

Laccases (benzenediol oxygen reductases; EC 1.10.3.2) are multi-copper oxidases with the potential to simultaneously oxidize a range of aromatic compounds by converting molecular oxygen to water. They can catalyse both catabolic reactions and polymerization reactions at ambient conditions. The fact that they can use a wide range of substrates may make these enzymes interesting for biotechnological applications. Laccase enzyme has been widely used in environmental remediation (removing chemicals), biofuel cell biosensor, textile

(denim bleaching), pulp paper, such as food, organic synthesis, fiber board, cosmetics, and paint. From the applications, it appears that the most used source is *T. versicolor* [21].

In our study, we used *T. versicolor*, which is known as the best laccase producer. To achieve optimal laccase production with the *T. versicolor* isolate provided by Prof. Dr. Mustafa Yamaç (Eskişehir Osmangazi University), we performed our experiments as described in the Material and Method section.

As can be seen in Figure 1, optimal laccase production was achieved on day 6. In the studies conducted on optimal laccase production from *T. versicolor*, the incubation period varies between 5 and 20 days [22-24]. Variation in incubation time appears to depend on the medium used, inducers, aeration, type of fermentation (solid phase or liquid), and age of the inoculum source. The optimal time of day for laccase production determined in our study confirms this.

For the purification of the laccase enzyme from the crude extract, we chose the TPP technique because it is easy to handle, cost-effective, environmentally friendly, and provides a high enzyme yield [8]. Figure 2 exhibits that purification was achieved with a 9.5-fold purified protein and 93% enzyme recovery. In literature, either lower purification fold values or recoveries were observed. For example, Kumar et al. [25] purified laccase from *Pleurotus ostreatus* with 7.22-fold protein but 184% yield. In another study, the laccase enzyme was purified 13.9-fold with a yield of 59% [26]. Rajeeva et al. [1] managed to purify laccase from *Ganoderma sp* with 60% recovery and 13.2 purification fold.

V. CONCLUSION

In this study, the qualitative determination of laccase of *T. versicolor* was performed and it was found to possess guaiacol activity. It was observed that the laccase of *T. versicolor* reached the highest activity on day 6. After optimising the conditions for three-phase distribution, 9.5-fold purification and 93% enzyme recovery were achieved. From the results, it can be concluded that the optimal conditions for separation, purification, and recovery of laccase from *T. versicolor* are as follows: 30% ammonium sulphate saturation (w/w), 1:1 ratio of crude extract to t-butanol (v/v), and pH 7.0.

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