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Short Communication

# Purification and Partial Characterization of a Phenol Oxidase from the Edible Mushroom *Auricularia Fuscosuccinea*

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## Abstract

A phenol oxidase from *Auricularia fuscosuccinea* was purified and partially characterized. Extracellular enzyme phenol oxidase was purified up to 55.9-fold from the culture filtrate by a protocol of three steps, ammonium sulfate precipitation twice (50 and 80% w/v), then two columns of ion exchange chromatography, first a DEAE-cellulose column and finally a high affinity resin column. The purified enzyme showed a molecular mass of 100 kDa, a $k_{cat}$ value of 2410 (±160) $min^{-1}$ and $K_M$ of 240 (± 30) mM when catechol is used as substrate. The enzyme showed a maximal activity of pH and temperature at 6.0 and to 40°C, respectively. The presence of ions ($Cu^{2+}$, $Na^{+}$, $Mg^{2+}$) did not improve the phenol oxidase activity. Inhibitors such as ascorbic acid and hydrazine, strongly affected the enzymatic activity. This is the first report on the partial characterization a phenol oxidase produced by the fungus *A. fuscosuccinea.*

**Keywords:** Bioremediation; Enzymes; Pollutants; White-Rot Fungi

## Introduction

Phenol oxidases are widely distributed among different organisms such as animals, plants, bacteria and fungi. White rot fungi are known as the most efficient organisms for lignin degradation. These fungi produce two major groups of ligninolytic extracellular enzymes; polyphenol oxidases and peroxidases [1]. Phenol oxidases (PO's) comprise a dinuclear copper center and some members of this family are: catechol oxidases, laccases, tyrosinases and cresolases. Phenol oxidases are involved in the oxidation of aromatic molecules to form unstable compounds (*o*-quinones), with the reduction of molecular oxygen to form water [2]. They are involved in different physiological activities such as the melanin synthesis, lignin degradation and the detoxification of phenolic molecules. In addition, PO's are relatively nonspecific and able to transform a variety of lignin-like aromatic molecules [3,4].

Phenols and aromatic amines, derived from various industrial activities such as pulp delignification, coal conversion, petrochemicals and textile dye bleaching, are considered as high impact pollutants [5]. The use of immobilized fungal PO for the treatment of phenol-containing polluted effluents has been proposed [6,7].

*Auricularia* is a genus of edible mushrooms that belongs to the family of white rot fungi and is well known for its nutritional value. Recently, antioxidant and immunomodulatory effects of proteins and polysaccharides produced from species of this genus have been reported [8]. Particularly, *Auricularia* spp. has been described as potential degrader of divers environmental pollutants [9,10]. Information about the extracellular enzymes produced by the genus *Auricularia* is scarce, and only few studies refer to the production and purification of their extracellular oxidative enzymes [11]. Thus, the aim of this work was purify and partially characterize properties of a phenol oxidase isolated from liquid cultures of *A. fuscosuccinea*, in order to evaluate its potential use in the remediation of toxic effluents.

## Materials and Methods

### Strain and Growth Conditions

The strain *A. fuscosuccinea* ECS-0210 from the mycological collection of El Colegio de la Frontera Sur (ECOSUR) was used. The organism was grown in a chemically defined medium with 0.2% glucose and 0.5% yeast extract. The fungus was transferred to agar plates (1.5% agar) and incubated in the dark at 26-28°C. After 7 days of incubation, five disks (5 mm diameter) of mycelium were used to inoculate 30 ml of the same medium without agar and incubated in the dark at 110 rpm at 26°C. After another seven days the mycelium was homogenized in a mixer and 90 ml were used to inoculate 1 l of liquid medium (pH 6.6) in 4 l flask and incubated in the dark at 110 rpm at 26-28°C.

### Enzyme Purification

After three days of fermentation, the culture was centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was separated and stored at -20°C for 24 h in order to precipitate polysaccharides. The supernatant was filtered twice through filter paper (Whatman® No. 1) and then through a syringe filter unit Millipore with 1.2 µm pore size. Proteins were precipitated in two steps at 4°C. First, ammonium sulfate was added to reach 50% saturation, and the solution was centrifuged at 12,000 rpm for 10 min. In the supernatant, the salt concentration was further increased to reach 80% saturation. After another centrifugation step, the precipitate was suspended in 10 mM phosphate buffer (pH 6.0) and dialysis was performed at 4°C for 24 hours, using a membrane (Sigma-Aldrich) with a 14 KDa molecular weight cut off. Finally, two types of anion-exchange chromatography protocols were performed, using a DEAE-cellulose column (30 x 2 cm) followed by a high affinity resin column (High Q; 10 x 2 cm) (Bio-Rad). The enzyme activity was monitored using syringaldazine (laccase substrate) and catechol (phenol oxidase substrate). The fraction showing phenol oxidase activity and no laccase activity was selected. Both columns were treated with the same buffer, and proteins were eluted applying a linear gradient of 0.0-1.0 M NaCl. 2.5 ml fractions were collected at a flow-rate of 2 ml/min. The fractions revealing phenol oxidase activity were united and concentrated by ultrafiltration (Amicon™) using a membrane filter with a pore size of 10 kDa (Millipore®).

### Enzyme Activity Assays

The phenol oxidase activity was measured by the oxidation of catechol to quinone ($\varepsilon_{436nm}$ = 3,450 $M^{-1}cm^{-1}$) [12]. The reaction mixture contained 100 mM catechol dissolved in phosphate buffer (100 mM, pH 7.0) and enzyme extract. The enzymatic activity using syringaldazine as a substrate was in the following reaction mixture: a phosphates buffer solution (100 mM, pH 6.6), syringaldazine 0.19 mM. The change in absorbance was monitored at 530 nm ($\varepsilon_{530nm}$= 64,000 $M^{-1}$ $cm^{-1}$) [13]. One unit (U) is defined as the amount of enzyme required for oxidizing 1 µmol of the substrate per minute.

### Gel Electrophoresis

For determining the molecular weight of the enzyme, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed, following the protocol by Laemmli [14] and stained with Coomassie-based Instant Blue™ (Expedeon)

### Protein Quantification

The protein content at different steps of the purification was determined by the Bradford method [15] using bovine serum albumin (BSA) as standard.

### Enzyme Activity Effectors

The effect of the temperature (10-60°C) and pH value (3.0-8.0) on the enzyme activity was determined using the same conditions as described above [12]. The substrate was added as reaction initiator. The buffers were prepared according to the value used, 100 mM acetate (pH 3.0-5.0) and 100 mM phosphate (pH 6.0-8.0). The effect of several inhibitors like ascorbic acid, hydrazine and various metal salts ($CuSO_4$, $MgSO_4$, and NaCl) at concentrations of 1 and 10 mM was determined at optimal values of pH and temperature. An analysis of variance (ANOVA) and means separation by the Tukey´s test ($P<0.05$) were performed with the software JMP ver. 6.0 (SAS Institute 2005).

### Kinetic Constants of Phenol Oxidase

The kinetics constants, $k_{cat}$ and $K_M$, of the purified preparation were determined using catechol as substrate in 100 mM phosphate buffer (pH 7.0). The substrate concentrations varied from 0 to 1000 mM. The purified PO from *A. fuscosuccinea* ECS-0210 fitted the Michaelis-Menten kinetics using MMfit software.

## Results

### Purification of the Phenol Oxidase

Phenol oxidase activity in the fungus *A. fuscosuccinea* ECS-210 was monitored during seven days (Figure 1). Enzyme activity reached a maximum after 4 days, while the protein concentration gradually increased with a maximum at day 3 and then decreased until day seven. After the second precipitation, the 39.7% of the PO activity was recovered with a 1.5-fold purification factor (Table 1). Most of the brown pigments or contaminant proteins were removed by DEAE-cellulose chromatography, as it was observed in the absorption profile of proteins present at 280 nm (Figure 2). After the high-affinity column (High Q) resulted in a single peak of both protein ($A_{280}$) and PO activity. The purified preparation showed a specific activity of 212.3 U/mg, representing 55.9-fold purified enzymatic preparation. This purified fraction does not show neither laccase activity using syringaldazine nor tyrosinase activity with L-tyrosine, while showed significant phenol oxidase activity with catechol as substrate. The purity of the enzyme was visualized by SDS-PAGE and we observed a single band of 100 kDa (Figure 3).

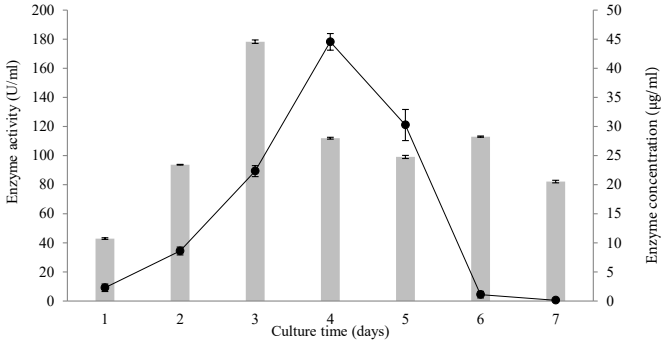


**Figure 1.** Phenol oxidase activity (•) and protein content (gray bars) of the extracellular medium from *A. fuscosuccinea* ESC-0210 cultures. The data represent the means of three replicate assays ± standard deviation.

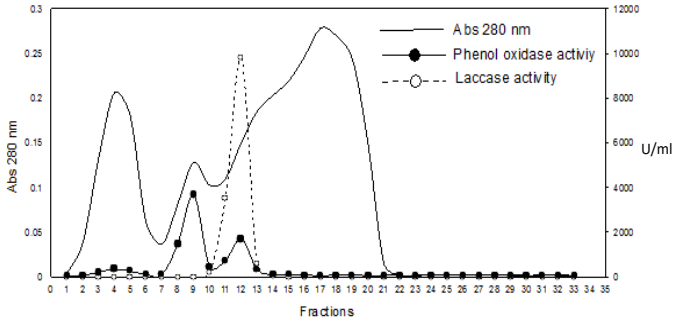


**Figure 2.** Anionic exchange chromatogram showing different elution profile for laccase and phenol oxidase activities.

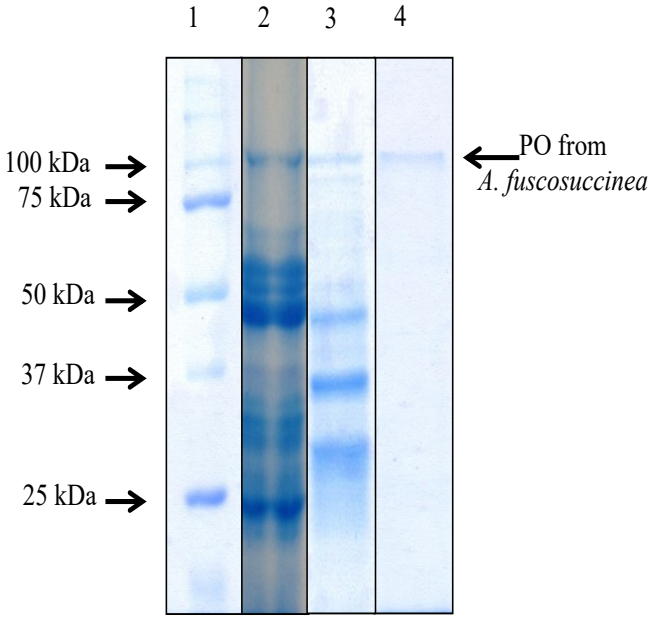


**Figure 3.** SDS-PAGE electrophoresis gel of the phenol oxidase isolated from *A. fuscosuccinea* ECS-0210. Well 1, molecular mass protein marker; Well 2, extract after $(NH_4)_2SO_4$ precipitation; Well 3, phenol oxidase fraction obtained after DEAE-cellulose chromatography; and Well 4, purified phenol oxidase after High Q chromatography.

**Table 1.** Activity and yield from the different steps during the purification protocol of phenol oxidase from *A. fuscosuccinea* ECS-0210.

| Purification step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Fermentation | 169 000 | 44.1 | 3 800 | 100 | 1 |
| Ammonium sulfate (80%) | 67 060 | 12.0 | 5 600 | 39.7 | 1.5 |
| DEAE–cellulose | 20 060 | 1.02 | 19 700 | 11.9 | 5.2 |
| High affinity resin (High Q) | 2 125 | 0.01 | 212 300 | 1.3 | 55.9 |

### Effect of Temperature, pH and Kinetic Constants

Temperature- and pH-activity profiles were determined for the purified PO preparation (Figure 4). The maximal PO activity was found at 40°C (Figure 4a) with a drastic activity decrease at 60°C. On the other hand, PO showed activity in a pH range of 3.0 to 8.0, with a clear optimal pH value of 6.0 (Figure 4b). The kinetic constants obtained were $k_{cat}$ of 2413 (±159) $min^{-1}$ and an affinity constant ($K_M$) of 241(±31) mM.

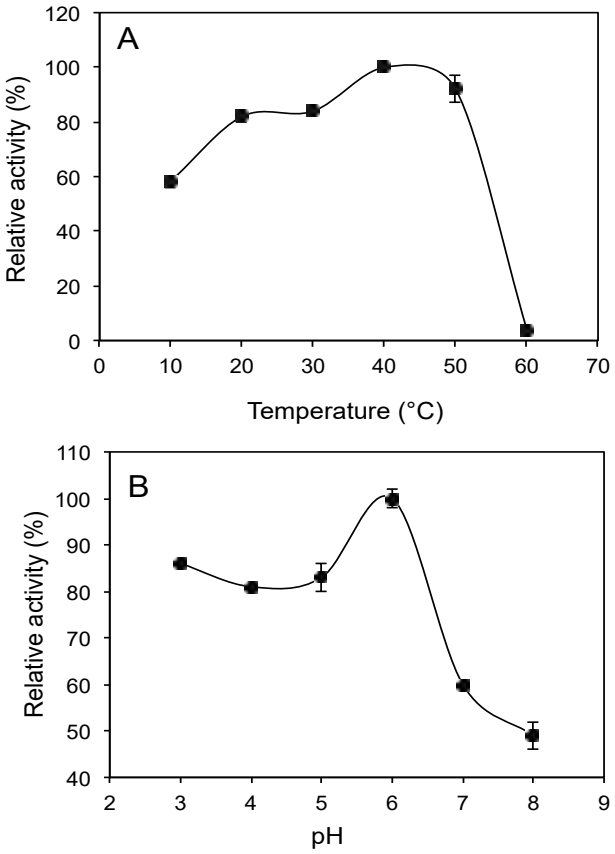


**Figure 4.** (A) Temperature activity profile of purified phenol oxidase from *A. fuscosuccinea* ECS-0210. Relative activity to 100% obtained at 40ºC and pH 6.0 with 100 mM catechol. (B) pH activity profile. A 100 mM acetate buffer was used for pH 3.0-5.0 and 100 mM phosphate for pH 6.0-8.0. Relative activity to 100% obtained at 26ºC with 100 mM catechol as substrate. Data represent mean values from duplicate assays ± standard deviation. Same letter are not significantly different (ANOVA, Tukey test, P<0.05).

### Effect of Inhibitors and Metal ions on the Phenol Oxidase Activity

We determined the influence of a range of phenol oxidase inhibitors and metal ions on the enzymatic activity (Table 2). The presence of ascorbic acid and hydrazine resulted in a 100% activity loss. Most of the metal ions showed no significant effect on enzyme activity. For the concentration of metal ions used, the enzyme activity was not significantly affected.

| Compound | Final concentration (mM) | Relative activity (%) |
|---|---|---|
| Ascorbic acid | 1 mM | 0 |
| | 10 mM | 0 |
| Hydrazine | 1 mM | 9 (±3) |
| | 10 mM | 7 (±3) |
| NaCl | 1 mM | 99 (±1) |
| | 10 mM | 100 (±3) |
| $CuSO_4$ | 1 mM | 113 (±3) |
| | 10 mM | 113 (±3) |
| $MgSO_4$ | 1 mM | 97 (±3) |
| | 10 mM | 93 (±3) |

**Table 2.** Effect of various compounds on the phenol oxidase activity of *A. fuscosuccinea* ECS-0210. Data represent mean values of duplicate assays ± standard deviations. Relative activity to control activity reaction (100%) in 100 mM phosphate buffer (pH 6.0). Control reactions were carried out always in parallel.

## Discussion

In this study, the phenol oxidase produced by *A. fuscosuccinea* ECS-0210 in submerged culture was purified and partially characterized. The phenol oxidase had a specific activity of 212.3 U/mg and was purified 55.9-fold. The success of a purification process depends on the response of the fungus to culture conditions and the techniques established for the purification protocol [12,16]. Recently, a tyrosinase produced by the fungus *Auricularia auricula* was described, using a three-stage protocol: ammonium sulfate precipitation, Sephadex G-100 and chromatography on DEAE-Sepharose, obtaining 27% yield and 21-fold purified enzyme [11].

The purity of the enzyme was confirmed as a single band of 100 kDa in SDS-PAGE electrophoresis. The molecular weight of PO from *A. fuscosuccinea* ECS-0210 is consistent with other fungal PO reported having molecular weight ranging from 38 to 150 kDa [17]. PO's produced by two strains of the fungus *Scytalidium thermophilum* showed molecular masses of 83 and 87 kDa [12,18], while plant PO's showed a range of 50–380 kDa [19,20].

*A. fuscosuccinea* PO shows an affinity to catechol close to the one reported for PO from *S. thermophilum* (with a $K_M$ value of 302.8 mM, [18], because the value of the kinetic constants $K_m$ and $K_{cat}$ were 241±31 mM and 2413±159 $min^{-1}$, respectively.

The maximal PO activity was found at 40°C and optimal pH val-

ue of 6.0. Fungal PO's are reported with optimal activities ranging from 6.0 to 7.5 and at temperatures of 55–65°C [12,18,21]. Finally, the effect of inhibitors and metal ions on enzyme activity was tested and observed that the phenol oxidase activity of *A. fuscosuccinea* is stable against the ions tested and ascorbic acid and hydrazine have a negative effect on enzyme activity. A similar effect of these compounds has been reported on the activity of the laccases from *Boletus erythropus* [12] and *Fomes fomentarius* [22].

## Conclusion

This is the first report on the biochemical characterization of a phenol oxidase produced by the fungus *A. fuscosuccinea*. This data could be the basis to explore its biotechnological potential for the transformation of phenolic pollutants.

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