

# Purification of Intracellular Laccase from *Schizophyllum sp.* Isolated from Forest Source

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**ABSTRACT:** Laccase enzymes extensively produced by white-rot fungi are evaluated in the field of biotechnology research due to its unique properties such as high reaction rates, broad substrate specificity and oxygen as an economical co-factor. The intracellular and extracellular laccase was obtained from white-rot fungus *Schizophyllum sp.* by liquid state fermentation. The intracellular laccase possess higher laccase activity in the 8<sup>th</sup> day of reaction and showed a decline from the 10<sup>th</sup> day. The isoenzymes were purified by ion-exchange and size exclusion chromatography. The native gel analysis rendered only one single band in the purified intracellular culture filtrate.

**KEYWORDS:** Laccase, *Schizophyllum sp.*, intracellular laccase, laccase activity, chromatography.

## I. INTRODUCTION

Laccases (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) belongs to the group of blue multicopper oxidases (MCO) which seems to represent the largest subgroup of MCO possessing distinct redox potential of copper ions to catalyze the one-electron oxidation of various aromatic substrates and phenolic compounds and concomitantly reduce molecular oxygen to water (Solomon et al., 1996, Messerschmidt, 1997). So far analysis reported that Laccases have been mostly found in all wood rotting fungi analyzed (Heinzkill, 1997).

Laccase enzyme is secreted by most of the lignin degrading basidiomycetes and it has been reported as an essential enzyme for lignin degradation in fungi. The abundant presence of common wood root fungus *Schizophyllum commune* in decayed woods specifies its responsibility in lignin degradation. The extracellular enzyme production and the growth of laccase producers are mostly affected by heavy metals (Baldrian et al., 2000; Pointing et al., 2000). Enzymes mostly exist in the crude form which has to be purified for further use. Generally, plant enzymes are purified from the sacs or tissues and microbial enzymes are purified from the production medium. Laccase purification is typically carried out by means of ammonium sulphate, ethanol precipitation (Hess et al., 2002; Viswanath et al., 2008) followed by ion exchange chromatography / size exclusion chromatography (Han et al., 2005; Cordi et al., 2007).

In this present work, the new Laccase strain *Schizophyllum sp.* and the intracellular laccase was identified. The intracellular fungal laccase was subjected to production, purification and molecular characterization.

## II. MATERIALS AND METHODS

### 1. Isolation of Microorganism

The isolation of positive laccase producers were carried out from the samples collected from forest soil, decomposed leaves and decayed wood. The samples were collected in sterile bags and cultured on potato dextrose agar incorporated with 0.2mM Guaiacol. After testing, the laccase positive strains were subcultured to obtain pure culture. The pure

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Vol. 5, Issue 4, April 2016

cultures were again tested for laccase activity with Guaiacol. Laccase oxidizes Guaiacol to deep red complex which indicates the presence of enzyme.

## 2. Cultivation in Liquid Medium

Laccase producers were cultivated in the growth medium containing 2 g yeast extract, 0.75 g  $\text{NH}_4\text{Cl}$ , 2 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.5 g KCl, 0.05g  $\text{CuSO}_4$  and 0.2 g thiamine in 20mM acetate buffer (pH 4.5). The mycelia pellets were inoculated into the culturing medium and were maintained under constant shaking condition at a rate of 120rpm for 15days. Laccase activity was measured from the first day of inoculum to 20 days.

## 3. Extraction of Intracellular Laccase

Intracellular enzymes were extracted following the modified method of Shilpi et al., 2007. After the desired growth, mycelial balls were collected and suspended in 50 mM acetate buffer (pH 4.5) containing 0.1% Triton X-100 for 1hr at 25°C to recover enzyme bounded to the cell and centrifuged at 10,000 g for 15 min. The pellets were collected and wash with sterile double distilled water and crushed using sterile glass wool in a pre-chilled mortar and pestle with the addition of 20 mM acetate buffer (pH 4.5) containing 1 mM EDTA, 2 mM 2-mercaptoethanol. The pellets were then washed twice with ice-cold water, crushed with sterile glass wool using chilled mortar and pestle in the presence of 20 mM acetate buffer (pH 5.0) containing 1 mM EDTA, 2 mM 2-mercaptoethanol. The suspensions were centrifuged at 15,000 g for 20 min. Unbroken cells and cell debris were removed by centrifugation at 32,000 g for 30 min. The supernatant was collected and dialyzed against 100 mM acetate buffer (pH 4.5) for 24 h and used as the source of intracellular enzyme.

## 4. Laccase Assay

Laccase activity was measured spectrophotometrically as described by Wolfenden and Wilson (1982) with 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as substrate. The reaction mixture consists of 1ml of 1mM ABTS in 0.1mM Sodium acetate buffer (pH 5.0) and 0.1ml of extracted enzyme. The reaction was monitored by measuring the oxidation ability at absorbance 436nm after for every 5 minutes of incubation. One unit of enzyme activity was determined by the moles of ABTS oxidized per minute by the enzyme at 25°C.

## 5. Purification

Laccase purification was performed based on the method of Benny *et al.*, 1998. The dialysed samples were loaded onto a DEAE-Cellulose anion-exchange column 18 x 1.5 cm, 0.1M Sodium acetate buffer (pH 4.5) were used for equilibration by linearly increasing NaCl concentration gradient (0 to 0.5 M) in the same buffer. The 10 fractions containing laccase activity were pooled, concentrated and dialyzed overnight against same buffer. Gel filtration chromatography was performed using Sephadex G-100 column 100 x 2.5 cm. The packed gel column was equilibrated with 0.1M sodium acetate buffer pH 4.5. Concentrated enzyme was loaded on to the Sephadex G- 100 column and eluted with 0.1M sodium acetate buffer (pH 7.0) and the DEAE-purified sample was loaded on to the column at the flow rate of 3ml per 15 min.

## 6. Gel Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). SDS-PAGE was carried out on a 4% w/v stacking gel and 10% w/v separating gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue for visualization of bands.

## 7. Zymography

The intracellular activity was detected based on the modified method of Tellez-Tellez *et al.*, 2005. Native PAGE gel (12%) was prepared using 100 g acrylamide/L and 27 g bis-acrylamide/L containing the running gel and 37g acrylamide/L and 50 g bis-acrylamide/L containing stacking gel. Sephadex purified enzyme 30 $\mu\text{l}$  was mixed with sample buffer without SDS as a reducing agent. The samples were loaded on to the gel without heating in the 12% gels of electrophoresis supplied with 100 V for 1-2 hrs. A substrate solution was prepared with acetate buffer (pH 5.5)

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 4, April 2016

containing 2mM of Guaiacol. Finally, the gels were placed in the substrate solution at room temperature for 1h till the appearance of laccase activity bands obtained by the oxidation of substrate.

## III. RESULTS AND DISCUSSION

### 1. Isolation of Microorganism

Laccase producers were screened with Chromogen Guaiacol, a sensitive substrate. Of the 10 isolated cultures, a single culture was observed to oxidize Guaiacol and considered to be a positive culture and microscopic view shows thin hyphae, septate with clamp connection (Fig. 1). Growth in Potato Dextrose Agar showed the development of white aerial cottony mycelium after 10-15 days of incubation which was identified as *Schizophyllum sp.* The microscope examination by Tullio et al., 2008 revealed the presence of hyaline, septate hyphae with clamp connections and short, thin spicules in *Schizophyllum commune*. The culture filtrate of *Schizophyllum sp.* rendered Laccase activity under submerged condition.

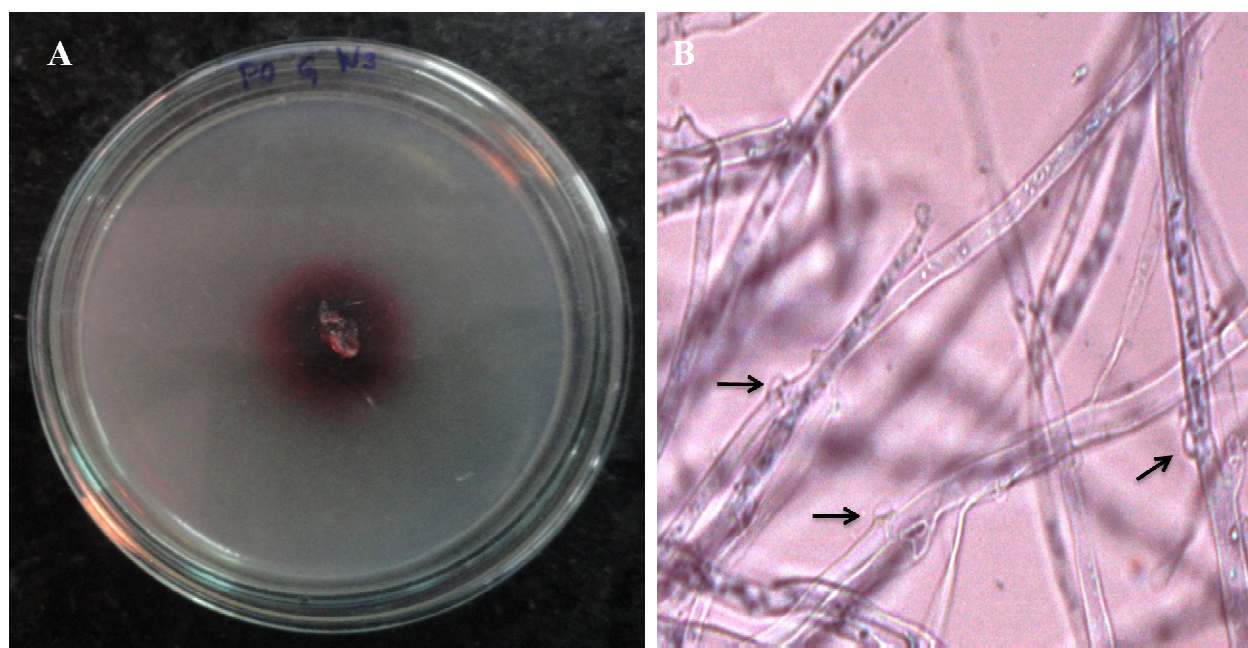


Fig.1 A: Laccase activity of *Schizophyllum sp.* in solid media incorporate with guaiacol B:Microscopic image of *Schizophyllum sp.* hyphae indicates clamp formation

### 2. Cultivation in Liquid Medium and Extraction of Intracellular Laccase

The *Schizophyllum sp.* cultured in the liquid medium indicated bead formation. Subsequent increase in growth, size and appearance of beads was reported from the day of incubation. The beads appeared to be extremely smooth in nature. The *Schizophyllum sp.* produced both extracellular and intracellular laccases. Most laccase studied are extracellular proteins although some intracellular laccase have been determined in some fungi and insects (Madhavi and Lele, 2009).

### 3. Laccase Assay

The extracellular laccase showed a maximum activity about 33 U/ml on the 10<sup>th</sup> reaction day when compared to that of the intracellular laccase. Intracellular laccase was found to be active from the 4<sup>th</sup> day of activity reaction till the decline stage of reaction. The intracellular laccase rendered a maximum activity on the 8<sup>th</sup> day (40 U/ml, Fig. 2). The results corroborated with the findings of Shilpi et al., (2006) whereby the Intracellular laccase activity of *T. clypeatus* was found to vary within the range of 1.50 -1.90 U/mg protein during this phase. The specific activity of intracellular

## International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 4, April 2016

laccase started to decrease from day 10 with the appearance of extracellular laccase activity in the culture filtrate. Palmieri et al., (2000) suggested that *Pleurotus ostreatus* was responsible for laccase production along with extracellular protease activity which disappeared in the early stage of growth.

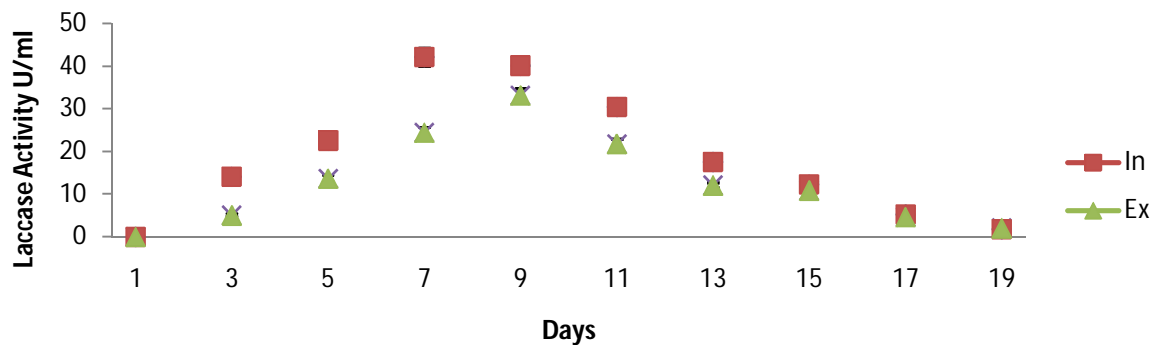


Fig 2: Laccase activity of *Schizophyllum* sp. Ex: Extracellular culture filtrate In: Intracellular culture filtrate. Error bar with standard deviation

#### 4. Gel Electrophoresis

The 12<sup>th</sup> day extracellular culture filtrate (80µg protein) of *Schizophyllum* sp. and intracellular culture filtrate (80µg protein) was analysed using 12% SDS. Large number of protein bands was visualized in intracellular culture filtrate than that of the extracellular culture filtrate. However, the purified samples on SDS-PAGE investigation showed single laccase isomer (data not shown). The appearance of large number of bands in the intracellular culture filtrate during the 8<sup>th</sup> reaction day might be due to the thrashing decrease in the laccase production and increase in the mycelial growth (Fig. 3). The enzyme active fractions were pooled, concentrated by lyophilization, dialysed against sodium acetate buffer (10mM; pH 5.5) and used for further purification. The purified intracellular enzyme resulted in 5.3 fold of purification with 53.1% of laccase enzyme yield and extracellular enzyme rendered only 2.2 purification fold with 30.9% yield. Hardik et al., (2014) reported an approach of *Pleurotus ostreatus* HP-1 laccase resulted in 13.13-fold purification with 77.63% yield of laccase enzyme DEAE - Sepharose. Diaz et al., (2010) reported 32.7 and 31.2 purification fold with 2.2 and 2.6 % yield for Lac I and Lac II isoenzymes from *Coriolopsis rigida*.

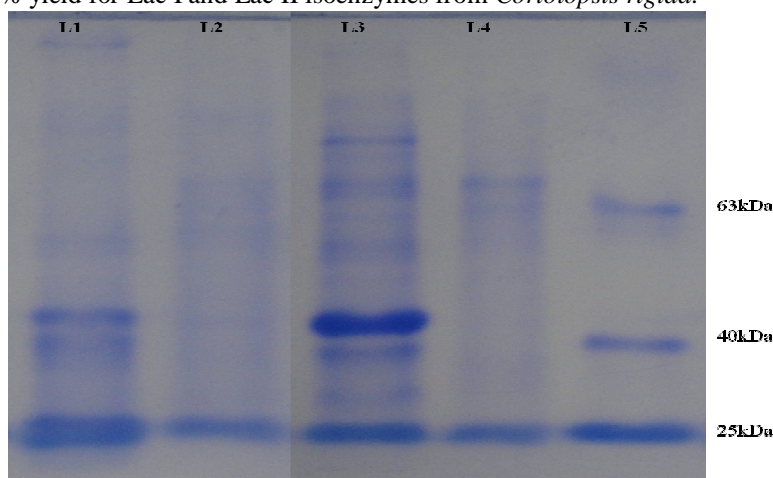


Fig 3: SDS PAGE analysis of *Schizophyllum commune* LU 207 culture filtrate. L1 - 12<sup>th</sup> day Intracellular culture filtrate, L2 - 12<sup>th</sup> day Extracellular culture filtrate, L3 - 8<sup>th</sup> day Intracellular culture filtrate, L4 - 8<sup>th</sup> day Extracellular culture filtrate, L5 – Molecular marker



## International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 4, April 2016

### 7. Zymography

Native PAGE analysis activity staining with chromogen Guaiacol stain of intracellular (20 $\mu$ g protein) and extracellular purified enzyme (20 $\mu$ g protein) showed the appearance of only one single band in both culture filtrate. The purified intracellular culture filtrate revealed highly stained laccase but the slightly active bands were visualized in the extracellular culture (Fig. 3). Pandeng et al., (2014) reported single protein band with laccase activity stained with ABTS as oxidation substrate. Dong and Zhang (2004) isolated two laccases viz., Lac I and Lac II from *Trametes gallica*.



Fig 4: Native PAGE analysis stained with 2mM Guaiacol. Lane I –Purified 8<sup>th</sup> day Intracellular culture filtrate, Lane II– Purified 8<sup>th</sup> day Extracellular culture filtrate

### IV. CONCLUSION

The culture filtrates were purified by two-step purification methods from the fungi *Schizophyllum sp.* which consequently resulted in the form of single isomers. Increased laccase activity was declared on the 5<sup>th</sup> day of reaction in the extracellular culture filtrate whereas it was found to be on 12<sup>th</sup> day with the intracellular culture filtrate. The purified laccase activity resolved *Schizophyllum sp.* as exogenic and intragenic laccase producers.

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(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 4, April 2016

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