Production of Extracellular Laccase by Lignin-degrading Basidiomycete *Coriolus versicolor* CV3

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리그닌 분해균 *Coriolus versicolor* CV3에 의한 Laccase의 생산

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SUMMARY

The cultural conditions in shake flasks were investigated under which maximum amounts of laccase produced by a strain of white-rot fungus *Coriolus versicolor* CV3. The enzyme yields on potato-malt extract medium by the fungus were higher than on other media consisted of onion infusion or malt extract, with maximum activity of 1.50 unit/ml culture or 119.5 unit/g mycelium at 11 days of incubation. Maximum yields of laccase and growth were obtained by supplementation of yeast extract or potassium nitrate to the potato-malt extract medium. Addition of 2.5-xylidine at $4 \times 10^{-4}$ M concentration to the medium induced the laccase production 3.1-fold higher than the basal level, while the mycelial growth was somewhat repressed. The pH optimum for the growth and laccase formation by the fungus was between pH 4 to 4.5.

INTRODUCTION

Due to the diversity of the enzyme systems and mechanisms among lignin degrading microorganisms, phenol oxidases especially laccase (benzenediol : oxygen oxidoreductase, EC 1. 10. 3. 2, formerly O$_2$ : p-diphenol oxidoreductase) from various fungi have been studies for their production, purification and enzymatic properties. The first step in the studies of lignin biodegradation has been to cultivation conditions under which a rapid lignin degradation takes place and under which large amounts of lignin degrading enzymes are produced. The importance of an easily metabolized carbon source in addition to lignin has been realized as the importance of proper nitrogen [20,24,25], inducer [2,5,14] and oxygen levels [4,18]. An increased understanding of cultural conditions will definitely lead, in the future, to a more rapid expansion of knowledge of enzymes and mechanisms involved in lignin degradation. In the present work, attempts were made to investigate the cultural conditions

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for laccase production by a strain of white-rot fungus *Coriolus versicolor* which was selected and maintained in the Biological Chemistry Laboratory.

**MATERIALS AND METHODS**

**Fungal Strain**

The strain of *Coriolus versicolor* CV3 which was selected for its laccase activity among the various white-rot fungi and maintained in the Biological Chemistry Laboratory, Department of Agricultural Chemistry, was used throughout the experiments.

**Media**

The organism was maintained on potato-dextrose agar slants or plates. For the enzyme production and growth experiments, potato-malt extract broth was used and the other broths of potato-dextrose, onion-dextrose-peptone, malt extract-dextrose and malt extract-dextrose-peptone were employed for comparative purposes.

*Potato-Dextrose (PD) Agar* : Difco potato-dextrose agar (3.9%) was prepared and sterilized in 400 ml quantities, then dispensed into the appropriate volumes, approximately 20 ml quantities into petri dishes.

*Potato-Dextrose (PD) Broth* : Potato infusion (from 200 g), Difco Bacto dextrose (20 g) and Difco yeast extract (2 g) were dissolved per litre of distilled water.

*Potato-Malt Extract (PM) Broth* : Potato infusion (from 200 g) Difco malt extract (20 g) and yeast extract (2 g) were dissolved in distilled water to a final volume of 1 litre, then dispensed into the required volumes.

The infusion was prepared from fresh potatoes by peeling and slicing, boiling for 1 hour then filtering through the layers of cheese cloth.

*Onion-Dextrose-Peptone (ODP) Broth* [8] : Onion infusion (from 100 g), Bacto dextrose (20 g), Difco Bacto peptone (2 g), KH₂PO₄ (1.5 g), K₂HPO₄ (0.2 g), MgSO₄·7H₂O (0.5 g) and CaCl₂ (0.1 g) were dissolved per litre of distilled water.

Onion infusion was prepared by the same procedure as described for that from potatoes.

*Malt Extract-Dextrose (MD) Broth* [23] : Bacto dextrose (20 g), malt extract (20 g), yeast extract (2 g) were dissolved per litre of distilled water.

*Malt Extract-Dextrose-Peptone (MDP) Broth* [12] : Bacto dextrose (10 g), malt extract (10 g), Bacto peptone (2 g), yeast extract (2 g), KH₂PO₄ (2 g), MgSO₄·7H₂O (1 g), L-asparagine-hydrochloride (1 g) and thiamine-hydrochloride (1 mg) were dissolved per litre of distilled water.

**Cultivation**

The cultivations were conducted by batch, using shake flasks with volumes of 100 ml or 500 ml which contained 20 ml or 120 ml volumes of media, respectively. The mycelial culture which grown on PD agar plate for 7 days was cut in circles, 6 mm in diameter, with a borer designed for this purpose and the circles were inoculated into the flasks. Incubations were contained on a orbital incubator, Vindon Scientific Ltd., England, for the required periods at 28°C which operated at 125 rpm with a stroke diameter of 5 cm.

**Estimation of Growth**

The estimation of growth during cultivation was achieved by measuring the mycelial mass. The mycelial wads which lumped during shaking were harvested, washed, and dried at 80°C for 24 hours before weighing.

**Assay of Laccase Activity**

Laccase activity was determined spectrometrically by using syringaldazine as the substrate [19]. The assay was initiated by the addition of 0.01 ml of 4.47×10⁻³ M syringaldazine solution (1.6 mg/ml in methanol) to the mixture of 2.5 ml of citrate-phosphate buffer, pH 4.6, and 0.5 ml of culture filtrate which had been preincubated at 30°C for 3 minutes. The increase in the linear sector of absorbance was
measured at frequent intervals during first few minutes of reaction, using a Pye Unicam PU 8800 Spectrophotometer at the wavelength of 525 nm.

The activity of laccase was calculated from the following formula [15]:

\[ A = \frac{10^6 \cdot \Delta E}{\epsilon \cdot \Delta t} \]

where: \( \epsilon = 65000 \) (molar absorption coefficient) [6]
\( \Delta E = \) increase in absorbance at 525 nm
\( \Delta t = \) reaction time in seconds

Reducing Sugar Determination

Total reducing sugar was determined in the culture filtrate as described by Miller et al. [16] which originally developed by Sumner and Sisler [22], where they applied a modified DNA reagent containing Rochelle salts. The absorbance was read at 640 nm and compared with 3 standard curve constructed using glucose.

RESULTS AND DISCUSSION

Cultural Media

To optimize cultural conditions under which large amounts of laccase are produced by the strain Coriolus versicolor CV3 various media listed for white-rot fungi by previous investigators were tested together with nitrogen sources, initial pH and inducers.

Other than synthetic or defined media, the complex media consisted of potato infusion, onion infusion or malt extract were commonly used by many authors. In this experiment batch cultures in shake flasks were carried out employing the media based on potato, onion and malt extract plus glucose and yeast extract as described in Materials and Methods.

The mycelial growth and production of extracellular enzyme on these media were compared during incubation until the residual sugars were depleted. The results obtained were represented in Figure 1: A for level of extracellular laccase, B for mycelial growth, and C for residual sugars. As shown in the Figure, the fungus produced extracel-
Table 1. Production of extracellular laccase by cultures* of *Coriolus versicolor CV3 on various media

<table>
<thead>
<tr>
<th>Media</th>
<th>Day of maximum activity**</th>
<th>Laccase activity***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato-dextrose</td>
<td>11</td>
<td>0.98</td>
</tr>
<tr>
<td>Potato-malt extract</td>
<td>11</td>
<td>1.50</td>
</tr>
<tr>
<td>Onion-dextrose-peptone</td>
<td>9</td>
<td>0.60</td>
</tr>
<tr>
<td>Dextrose-malt extract</td>
<td>11</td>
<td>0.60</td>
</tr>
<tr>
<td>Dextrose-malt extract-peptone</td>
<td>9</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* Cultures of 20 ml per 100 ml flask were grown on orbital incubator as described in Materials and Methods.
** Days after inoculation.
*** Laccase activities are represented as mean values obtained from 6 replicate cultures.

Laccase on all the media examined with approximately a 2.5-fold variation in the maximum activity per unit volume of culture filtrate. The maximum levels of enzyme were also calculated in terms of the activity per unit weight of mycelium and presented in Table 1. In potato-malt extract medium the enzyme yields were higher than any other media comprised onion or malt extract, with maximum activity of 1.50 unit per ml of culture or 119.5 unit per gram of mycelium at 11 days of incubation. The maximum yield of enzyme was found at the mid of the exponential growth phase of the fungus on all the media employed. This was clearly shown in Figure 2, where the growth and laccase formation on PM were plotted in a graph together with the residual sugars in the culture. The reducing sugars were completely exhausted in 15 days. A marked decrease in residual sugar, from 9.1 mg/ml to 4.5 mg/ml, was observed during 9 to 11 days of incubation and this coincided with the period that the enzyme level reached the maximum. These results are contrasted to other works, where the best yields of laccase were attained on onion medium for *Pleurotus ostreatus* [13], and on malt extract medium for *Polyporus versicolor* [11], and *Agaricus bisporus* [23]. However it is generally unable to compare the levels of enzyme activity between different organisms, because the arbitrarily defined unit and the assay method vary with the investigator. The maximum laccase activity of *Pleurotus ostreatus* is shown as 1.2 x 10^-2 unit when grown on onion medium (double strength), but the data for mycelial growth are not available [13].

**Effect of Nitrogen Sources**

The preference of the fungus to nitrogen sources for laccase formation was examined by growing the strain on PM medium which contained peptone, urea, KNO₃, NH₄H₂PO₄ at concentrations of 0.05

![Fig 2. Laccase production, mycelial growth of Coriolus Versicolor CV3 on potato-malt extract medium.](image-url)
Table 2. Effects of nitrogen supplements to PM medium on laccase formation by *Coriolus versicolor* CV3

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Concentration</th>
<th>Laccase activity (unit/ml culture)</th>
<th>Dry weight of mycelium (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control without</td>
<td>none</td>
<td>0.97</td>
<td>5.4</td>
</tr>
<tr>
<td>Control with</td>
<td>0.05</td>
<td>1.38</td>
<td>6.0</td>
</tr>
<tr>
<td>Control with</td>
<td>0.2</td>
<td>1.50</td>
<td>6.3</td>
</tr>
<tr>
<td>yeast extract</td>
<td>0.05</td>
<td>1.19</td>
<td>5.2</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.2</td>
<td>1.30</td>
<td>6.0</td>
</tr>
<tr>
<td>Urea</td>
<td>0.05</td>
<td>0.86</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.58</td>
<td>4.2</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.05</td>
<td>1.27</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.44</td>
<td>5.5</td>
</tr>
<tr>
<td>NH₄H₂PO₄</td>
<td>0.05</td>
<td>1.16</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.26</td>
<td>5.4</td>
</tr>
</tbody>
</table>

The yeast extract in PM medium was substituted by various nitrogen sources at concentrations of 0.05 and 0.2%. The results of 11 days culture are presented.

and 0.2% instead of yeast extract. The enzyme production and mycelial growth were analysed and the results of 11 days culture are given in Table 2.

Maximum yields of laccase were obtained by addition of yeast extract or potassium nitrate, however the nitrogen supplements except for urea to PM medium appeared to support the growth and enzyme production of the fungus with a similar level. Urea supplement resulted in decrease of the enzyme level lower than that from PM medium without nitrogen. On the other hand nutrient nitrogen are known to affect the substantial degradation of wood lignin significantly [9,11,24], and ligninolytic enzyme synthesis by *Phanerochaete chrysosporium* in response to nitrogen starvation was reported by Keyser et al. [10].

**Effect of Initial pH**

The fungus was grown on PM medium which adjusted to pH range 3 to 7 and the enzyme yields and mycelial growth were determined. The results of 11 days culture shown in Figure 3 illustrate that pH optimum for the growth and laccase formation is between pH 4 to 4.5.

Most of white-rot fungi were reported to have an optimal pH for laccase formation at acidic range: pH 4.4–4.5 for *Phanerochaete chrysosporium* [10], pH 5 for *Polyporus versicolor* [3], and pH 5.5 for *Schizophyllum commune* [21]. And the substantial degradation of wood lignin was also optimized at acidic pH: pH 3-5 for *Coriolus hirsutus* IFO 4917 and 6477 [25], and pH 5.5 for *Pleurotus ostreatus* [7].

![Graph](image)

**Fig 3.** Effect of initial pH on laccase formation. The fungus was grown on potato-malt extract medium which adjusted to pH range 3.0–7.0, and the enzyme yields and mycelial growth were determined.
Table 3. Induction of laccase with phenolic compounds and transcription inhibitor

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Concentration</th>
<th>Laccase activity (unit/ml culture)</th>
<th>Dry weight of mycelium (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>none</td>
<td>1.50</td>
<td>6.4</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>1.3×10⁻⁶</td>
<td>1.44</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>2.6×10⁻⁶</td>
<td>2.60</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>5.2×10⁻⁶</td>
<td>2.32</td>
<td>2.9</td>
</tr>
<tr>
<td>o-Toluidine</td>
<td>1×10⁻⁴</td>
<td>1.54</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>2×10⁻⁴</td>
<td>2.52</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>4×10⁻⁴</td>
<td>3.50</td>
<td>4.8</td>
</tr>
<tr>
<td>2.5-Xylinolide</td>
<td>1×10⁻⁴</td>
<td>2.42</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>2×10⁻⁴</td>
<td>3.72</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>4×10⁻⁴</td>
<td>4.64</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The compounds were added PM medium after 3 days of incubation. The results of 11 days culture are presented.

Addition of Inducers

Some of phenolic compounds and transcription inhibitors reported for other fungi by previous investigators [2, 5, 17] were examined to ascertain whether extracellular laccase can be induced above basal levels by treatment of cultures of Coriolus versicolor CV3 with the compounds. Actinomycin D, o-toluidine and 2.5-xylinolide were tested by adding to the 3 days culture on PM medium. The results are represented in Table 3. Addition of actinomycin D, at 2.6×10⁻⁶ M concentration induced laccase production 1.7-fold higher than the basal level, o-toluidine at 4×10⁻⁴ M induced 2.3-fold and 2.5-xylinolide at 4×10⁻⁴ M induced 3.1-fold, whereas the mycelial growth was repressed. These results are somewhat similar to those obtained from Neurospora crassa [5] and Polyporus versicolor [2], but in contrast no positive effect of xylinolide or toluidine reported for Agaricus bisporus by Wood [23].

**적 요**

백색 부후군 Coriolus versicolor CV3 감주를 공시하여 laccase 생산을 위한 배양 조건을 검토하였다. 밀자, 양파 또는 맥아의 추출물을 주제로 한 각종 백색 부후균용의 배지를 비교한 결과, 공시 균주의 laccase 생산은 potato-malt extract 배지에서 가장 높아서 배양 11일 후 배양액당 1.50 unit/ml, 균체 건물 중당 119.5 unit/g 수준의 laccase 활성도에 도달하였다. potato-malt extract 배지에 각종 유기 및 무기 질소원 중 yeast extract 또는 KNO₃를 0.2% 증가하였을때 laccase의 생산 수준이 높았으며, inducer로서 2.5-xylinolide를 4×10⁻⁴M 증가한 경우 대조군에 비하여 3.1배 수준으로 laccase 생산이 유도 되었다. Laccase 생산을 위한 배양 최적 pH는 4~4.5 이었다.

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