Efficient Recovery of Lignocellulolytic Enzymes of Spent Mushroom Compost from Oyster Mushrooms, *Pleurotus* spp., and Potential Use in Dye Decolorization

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Abstract This study was conducted in order to perform efficient extraction of lignocellulolytic enzymes amylase (EC 3.2.1.1), cellulase (EC 3.2.1.4), laccase (EC 1.10.3.2), and xylanase (EC 3.2.1.8) from spent mushroom compost (SMC) of *Pleurotus ostreatus*, *P. eryngii*, and *P. cornucopiae*. Optimal enzyme recovery was achieved when SMCs were extracted with 50 mM sodium citrate (pH 4.5) buffer at 4°C for 2 hr. Amylase, cellulase, and xylanase activities showed high values in extracts from *P. ostreatus* SMC, with 2.97 U/g, 1.67 U/g, and 91.56 U/g, respectively, whereas laccase activity and filter paper degradation ability were highest in extracts from *P. eryngii* SMC, with values of 9.01 U/g and 0.21 U/g, respectively. Enzymatic activities varied according to the SMCs released from different mushroom farms. The synthetic dyes remazol brilliant blue R and Congo red were decolorized completely by the SMC extract of *P. eryngii* within 120 min, and the decolorization ability of the extract was comparable to that of 0.3 U of commercial laccase. In addition, laccase activity of the SMC extract from *P. eryngii* was compared to that of commercial enzymes or its industrial application in decolorization.

Keywords Activity, Decolorization, Lignocellulolytic enzymes, *Pleurotus* spp., Spent mushroom compost

*Pleurotus* spp., including *Pleurotus ostreatus* and *P. eryngii*, are mainly edible mushrooms that account for more than 60% of total mushroom production in Korea. The mushrooms are produced mainly on a commercial scale using automated systems, and their culture substrates include numerous lignocellulosic wastes, including corncobs, sugarcane wastes, cottonseed hull, cotton and beet pulp, and sawdust supplemented with rice bran in vinyl bags and bottles. In Korea, it is estimated that approximately 2 million tons of spent mushroom compost (SMC) is produced yearly by mushroom farms in Korea, with almost 1.2 million tons generated from oyster mushrooms. This massive amount of SMC is unsuitable for reuse in mushroom production, therefore, it is used either as garden fertilizer or deposited in landfills, which pollutes the environment. SMC is composed of fungal mycelia, extracellular enzymes secreted from mushrooms for degradation of substrates, and unused lignocellulosic substrates. SMC has been used in production of value-added products such as biogas [1] and bulk enzymes [2], for bioconversion into organic fertilizer [3], for use as animal feed supplements [4], and for degradation of pentachlorophenol [5]. These uses benefit human health and the environment because an agricultural waste forms the raw material for novel processes.

Basidiomycetes, such as oyster mushroom, king oyster mushroom, winter mushroom, and shiitake, can enzymatically degrade diverse substrates containing lignin, hemicellulose, and cellulose into soluble compounds of low molecular weight. These soluble compounds are then absorbed by fungal hyphae through a process called nutritive absorption. Three major groups of enzymes involved in breaking down
lignin and cellulose agro-wastes are cellulases, xylanases, and laccinases. These extracellular enzymes have been used in the brewing, baking, starch-processing, leather, and textile industries. Cellulase and xylanase have recently been used as the main enzymes in saccharification of biomass for production of biofuel. In addition, lignin degrading enzymes such as laccases have been used in decolorization of industrial synthetic dyes such as remazol brilliant blue R (RBBR), Congo red, and indigo [6, 7].

In this study, optimal extraction conditions for production of lignocellulolytic enzymes, including cellulase, xylanase, and laccases from SMCs of *Pleurotus* spp. were investigated and SMC extracts were further examined for industrial usefulness in decolorization of synthetic dye.

### MATERIALS AND METHODS

**SMC collection.** Post-harvest media from bottle-cultivations of *P. ostreatus* were collected from six farms in the Gyeonggi-do region of Republic of Korea. SMCs of *P. eryngii* and *P. cornucopiae* were provided by the mush-heart mushroom farm and Mushroom Research Institute (Gwangju, Korea). SMC was collected immediately after harvest of mushrooms and lyophilized and later used for extraction of extracellular enzymes. The dried SMC samples (10 g) were suspended in 50-mL buffer.

**Extraction of extracellular enzymes from SMCs.** Extracellular enzymes were extracted using four solutions from *P. ostreatus* SMC: tap water, distilled water, 50 mM sodium citrate buffer (pH 4.5), and 50 mM sodium phosphate buffer (pH 8.0). The SMC-buffer mixtures were incubated with shaking at 200 rpm for 2, 4, 6, 8, 10, or 12 hr at 4°C or 20°C. Each sample was filtered through miracloth (pore size: 22–25 μm) and then centrifuged at 10,000 × g at 4°C for 15 min. The supernatant, which constituted the crude enzyme extract, was assayed.

**Enzyme assays.** Enzyme activities are represented as U/g substrate. Protein concentration was determined using the Bradford method [8] with bovine serum albumin standard. All assays were performed in triplicate for each extraction medium. Amylase (EC 3.2.1.1) activity was measured using the 3,5-dinitrosalicylic acid (DNS) reagent method described by Miller [9]. Ten microliters of SMC extract was added to 90 μL of 1% starch in distilled water and incubated at 50°C for 60 min, followed by addition of 100 μL of DNS to stop the reaction. The solution was boiled for 10 min and then placed on ice for 5 min. The amount of reducing sugar was determined using the DNS method with maltose as standard by measuring absorbance at 575 nm using a spectrophotometer (SUNRISE; Tecan, Grödig, Austria); 1 U of amylase was defined as the amount of enzyme required for release of 1 μmol of reducing sugar in maltose equivalents per min. Cellulase (EC 3.2.1.4) was measured using carboxymethyl cellulose (CMC) as the substrate [10]. Ten microliters of SMC extract solution was added in 90 μL of 0.25% CMC solution and reacted at 50°C for 60 min, and then measured using the DNS assay at 575 nm with glucose as a standard [11]; 1 U of CMCase was defined as the amount of enzyme required for release of 1 μmol of reducing sugar in glucose equivalents per min under the assay conditions described above. Xylanase activity was estimated using a 1% (w/v) suspension of Xylan from beech wood as the substrate. A mixture composed of 90 μL of 1% xylan (Sigma, St. Louis, MO, USA) and 10 μL of SMC extract was reacted at 40°C for 30 min and the amount of reducing sugar was determined using the DNS method at 575 nm with D-xylose as a standard. Enzyme activities are expressed in units, where 1 U is defined as the amount of enzyme required for production of 1 μmol product/min, and productivity is presented as U/g of the SMC. Laccase activity was assayed by measuring the oxidation of 2,2’-azinobis[3-ethylbenzothiazolone-6-sulfonic acid] (ABTS). SMC extract (10 μL) was mixed with 90 μL of 50 mM sodium acetate buffer (pH 4.5) containing 1 mM ABTS and then reacted at room temperature. The reaction was stopped by addition of 100 μL of 20% (w/v) trichloroacetic acid. Oxidation of ABTS was monitored at 420 nm on a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required for oxidation of 1 μmol/min of the substrate under the assay conditions.

**Filter paper (FP) assay for total cellulase activity.** Cellulase activity was determined using the method of Eveleigh et al. [12]. A 1-mL aliquot of SMC extract was transferred to a clean test tube, followed by addition of 1 mL of sodium citrate buffer (pH 4.8). A Whatman #1 filter paper strip (6 cm × 1 cm) was then added to each tube, which was vortexed in order to coil the filter paper at the bottom of the tube. Tubes were incubated in a water bath at 50°C for 1 hr, followed by addition of 1 mL of DNS reagent. The tubes were placed in a boiling water bath for 10 min and then in an ice-bath, and the reactions were stopped by addition of 1 mL of 40% sodium potassium tartarate to each tube. The contents of the tubes were mixed and absorbance was measured at 575 nm. Reducing sugar levels were determined using the DNS method with D-glucose as a standard at 575 nm.

**Dye decolorization by SMC extracts.** Dye-decolorization capacity of SMC extracts was monitored using 0.5% Congo red and RBBR at room temperature. An aliquot of SMC extract (25 μL) was incubated for various periods of time with 3 mL of 20 mM sodium acetate buffer (pH 4.0) containing 0.05% RBBR or Congo red at 25°C. Laccase (0.3 to 10 U/mL; Sigma) was used as a positive control. Dye decolorization was measured as change in absorbance at 595 nm using a spectrophotometer and then expressed in percentages. Negative-control experiments were performed under identical conditions without addition of SMC extract.
and all experiments were performed in triplicate. The decolorization percentage was calculated as follows: 
\[ \text{Decolorization percentage (\%) =} \frac{(A_0 - A)}{A_0} \times 100 \]
where \( A_0 \) is the dye absorbance of the control, and \( A \) is the dye absorbance of the test sample.

**RESULTS AND DISCUSSION**

**Enzymatic activity in SMC extracts of *P. ostreatus* prepared using distinct extraction buffers.** Enzyme activities were measured in SMC extracts of *P. ostreatus* prepared using four distinct buffers; the results are shown in Table 1. SMC extract contained 0.29–0.43 mg/g protein based on the buffer used, with the highest protein yield obtained with 50 mM sodium phosphate (pH 7.0). Recovery ratios of the enzymes from SMC were calculated for each buffer in order to confirm amylase, cellulase, xylanase, and laccase activities, and the profiles showed similar patterns for the four buffers for each of the enzymes studied (Table 1). The highest amylase activity (2.81 U/g) was obtained using the 50 mM sodium citrate (pH 4.5) extraction buffer, and the lowest (2.16 U/g) was obtained using 50 mM sodium phosphate (pH 7.0). The lowest CMCase activity (0.78 U/g) was detected in 50 mM sodium phosphate (pH 7.0), the highest (2.18 U/g) in 50 mM sodium citrate (pH 4.5). Laccase activity (1.95–2.23 U/g) differed only slightly from that of amylase. Recovery of xylanase activity was substantially higher than that of other enzymes: 49.80–58.30 U/g xylanase in the SMC extract. The lowest xylanase activity was detected in extracts prepared using 50 mM sodium phosphate (pH 7.0), and the highest activity was detected in extracts prepared using 50 mM sodium citrate (pH 4.5). The results showed that comparable amounts of the tested enzymes could be recovered using distinct extraction buffers and were in agreement with the findings of Bisaria *et al.* [1] and Singh *et al.* [13], who reported successful extraction of CMCases, xylanase, and β-glucosidase using 50 mM citrate buffer (pH 4.8) from the SMC of *P. sajor-caju* (Summer Oyster) grown on rice and wheat straw. However, in this study, the xylanase activity recovered was 6-fold higher than that reported by Singh *et al.* [13].

The effects of extraction time and temperature on enzyme productivity were also assessed using the SMC of *P. ostreatus*. SMC mixed with 50 mM sodium citrate (pH 4.5) was incubated for 2–12 hr at 4°C and 20°C. The recovery of xylanase did not show significant change with different time intervals and temperatures, although higher enzyme activity was observed with incubation at 20°C (Fig. 1). Additional conditions for increasing enzyme productivity were evaluated by homogenizing SMC. The substrate was prepared as described (see Materials and Methods) and 10 g of SMC was mixed with 50 mM sodium citrate (pH 4.5) extraction buffer and homogenized at speeds of 1,500, 3,000, 6,000, 8,000, 10,000, and 20,000 rpm. After homogenization, SMC extracts were prepared as described above and the cellulase activities in the extracts were compared with activities in extracts prepared with shaking incubation. The results of this study are in agreement with previous findings; enzyme extraction from the SMC of *P. sajor-caju* was maximal with 2–3 hr incubation at room temperature [13]. In conclusion, extraction of enzymes using a shaking incubator and tap water or 50 mM sodium citrate (pH 4.5) buffer at 25 ± 2°C (room temperature) for 2 hr is an economical method for extraction of substantial amounts of SMC enzymes of *P. ostreatus*.

Because solid state fermentation (SSF) mimics the conditions of the natural fungal growth, it is particularly suitable for production of enzymes using filamentous fungi [14]. Like SSF, use of mushroom substrates containing natural solid substrates (especially lignocellulosic agricultural residues as growth substrates) is useful for stimulating the production of enzymes such as cellulases, xylanase, and laccases. The presence of lignin and cellulose/hemicellulose induces enzyme

| Enzyme activity (U/g of SMC) | Tap water | DW | 50 mM sodium citrate (pH 4.5) | 50 mM sodium phosphate (pH 7.0) |
|-----------------------------|-----------|----|------------------------------|------------------------------|
| Protein (mg/g)              | 0.372 ± 0.0251 | 0.357 ± 0.0508 | 0.288 ± 0.0475 | 0.433 ± 0.0396 |
| Amylase                     | 2.2 ± 0.17 | 2.4 ± 0.18 | 2.8 ± 0.21 | 2.2 ± 0.27 |
| Cellulase                   | 1.20 ± 0.144 | 1.61 ± 0.113 | 2.18 ± 0.213 | 0.78 ± 0.053 |
| Laccase                     | 1.9 ± 0.26 | 2.0 ± 0.29 | 2.2 ± 0.30 | 2.2 ± 0.22 |
| Xylanase                    | 56 ± 9.3 | 55 ± 9.4 | 58 ± 9.3 | 50 ± 7.8 |

![Fig. 1. Effects of time and temperature on recovery of xylanase from *Pleurotus ostreatus* spent mushroom compost (SMC). The results are expressed as mean of three replicate samples.](image-url)
production and these compounds are rich in sugars that enhance fungal growth, making the process economical. SMC is likely the final product of SSF that is suitable for production of enzymes using mushrooms.

**Enzymatic activity in extracts of SMC collected from various mushroom farms.** The activities of amylase, CMCase, xylanase, and laccase were measured in extracts of SMC from six mushroom farms located in Geonggi-do in Korea; enzyme recovery varied markedly in the extracts of samples from these farms (Fig. 2). The highest amylase activity (3.87 U/g) was obtained from the SMC extract of mushroom farm C and was four times higher than the activity (0.95 U/g) in the SMC extract of mushroom farm A. The highest (3.59 U/g) and lowest (0.2 U/g) CMCase enzyme activities were measured in extracts of SMC from mushroom farms B and D, respectively. The highest activities of laccase (5.89 U/g) and xylanase (170.28 U/g) were measured in SMC extracts of mushroom farms C and B. Extracts from the B farm SMC showed the highest enzyme activity. Substrates used in oyster cultivation generally contain aspen and willow sawdust, cotton seed meal, beet pulp, rice bran, wheat bran, and sawdust. However, the substrate composition differed for each farm, and, thus, the variation in enzymatic activities measured could be due to distinct compositions of raw materials in the substrates used on the farms.

**Enzyme productivity in extracts of SMCs from different Pleurotus spp.** Enzymes were extracted from SMCs of *P. ostreatus*, *P. cornucopiae*, and *P. eryngii* and their activities were compared (Fig. 3). Amylase activity in the SMC extract of *P. ostreatus* (2.37 U/g) was nearly double that in extracts of *P. cornucopiae* (1.40 U/g) and *P. eryngii* (1.48 U/g). CMCase activity in SMC extract of *P. ostreatus* was 1.67 U/g, however, no activity was detected in *P. cornucopiae*, and was low in *P. eryngii* (1.23 U/g).

Cellulase activity was further monitored based on FP degradation. *Pleurotus* spp. SMC was extracted with 25 mL of 50 mM sodium citrate (pH 4.5) and the liquid enzyme was tested for FP activity (exo-1,4-B-D-glucanase). FP degradation for 1 hr at 50°C was confirmed using the reaction supernatant and DNS assay. According to the results, *P. cornucopiae* appeared to have little CMCase activity, but clearly had FP activity (0.12 U/g). In addition, CMCase and FP activities of *P. ostreatus* and *P. eryngii* were detected and, in particular, a high activity was observed for *P. eryngii* (Fig. 3). A previous study showed that the SMC of *P. eryngii* can digest FP [15] and the results indicated that the enzymes

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**Fig. 2.** Recovery of enzymes in spent mushroom compost (SMC) extracts of *Pleurotus ostreatus* from six different mushroom farms.

**Fig. 3.** Enzymatic activity in spent mushroom compost (SMC) extracts of *Pleurotus* spp. Each aliquot of SMC extracts from *P. eryngii* bar A, *P. ostreatus* bar B, and *P. cornucopiae* bar C was used for enzymatic activities of amylase, laccasae, xylanase, cellulase, and filter paper (FP) degradation ability.
extracted from the SMC of *Pleurotus* have industrial value. Thus, the results of this study showing that the SMC extracts of *P. cornucopiae* and *P. eryngii* can digest FP suggest that SMC can be used in production of bio-energy by degradation of biomass.

The activity of laccase (8.01 U/g) was highest in SMC extract of *P. eryngii* and the enzymatic activity was 3.77 U/g and 2.97 U/g in SMC extracts for *P. cornucopiae* and *P. ostreatus*. Xylanase activity (91.68 U/g) of *P. ostreatus* was 9-10-times higher than that of *P. cornucopiae* (8.06 U/g) and *P. eryngii* (12.85 U/g). These results supported the conclusion that high enzyme productivity depends on the unique characteristics of the mushroom species. Xylanase and laccase, which showed high activity in SMC extracts of *P. ostreatus* and *P. eryngii*, will be produced for industrial applications. Xylanases are enzymes found commonly in microorganisms, marine algae, protozoans, nannals, crustaceans, insects, seeds, plants, and other natural sources, however, the principal commercial source of these enzymes is filamentous fungi [16]. Xylan, also known as “wood gum,” is a gum-like polysaccharide found in plant cell walls. The basic structure of xylan comprises a linear backbone of 1,4-linked D-xylopyranose residues and a 5-carbon reducing sugar [17]. The major function of xylanase is to break down a type of fiber called hemicellulose by converting one of its components, (β-1,4) xylan, into a simple sugar called xylose. Thus, xylanase helps break down plant cell walls. Recently, xylan and its hydrolytic enzymatic complex have attracted considerable industrial interest as a supplement in animal feed, for the manufacture of bread, food, and drinks, and for use in textiles, bleaching cellulose pulp, and ethanol and xylitol production [16].

Laccase is the most widely distributed of all of the large blue copper-containing proteins present in diverse higher plants and fungi, and, recently, characterization of laccases in laccases in bacteria has also been reported [18]. Laccase belongs to a broad group of enzymes called polyphenol oxidases, which contain copper atoms in the catalytic center and are usually called multicopper oxidases. Laccases are similar to other phenol-oxidizing enzymes, which preferably polymerize lignin by coupling with the phenoxy radicals produced by oxidation of lignin phenolic groups. Fungal laccases have widespread applications, ranging from effluent decolorization and detoxification to pulp bleaching, removal of phenolics from wines, organic synthesis, manufacture of biosensors, synthesis of complex medical compounds, and dye-transfer blocking functions in detergents and washing powders [18].

**Decolorization of dyes by SMC extracts.** Two synthetic dyes were selected for evaluation of the dye decolorization potential of SMC from *Pleurotus* in comparison with commercial laccase. The two industrial synthetic acidic dyes, RBBR and Congo red, represent anthraquinone and diazo dyes, respectively, and they were used as decolorization substrates for evaluation of the dye-decolorization efficacy of laccase in SMC extracts. Among the SMCs of *Pleurotus* spp., *P. eryngii* SMC showed the highest decolorization activity, and *P. ostreatus* SMCs showed differences in decolorization depending on the farm from which the SMCs were collected (Fig. 4). Decolorization values varied for the two dyes, which may be due to structural and/or redox potential differences of the dyes. Of particular interest, SMC decolorized the diazo-like dye Congo red without any mediator, even though this dye is not a common substrate for the laccase catalytic reaction [6]. These results are in agreement with those previously reported on purified fungal laccases, which showed extremely high activity in decolorizing anthraquinone-like dyes [19] but reduced activity against azo-like dyes [20]. Before measurements, the full wavelength absorption spectra (from 400 nm to 800 nm) of the dyes were recorded, which gave the maximal spectral peak at 595 nm for RBBR (Fig. 5A). The SMC extracts were next used for measurement of the decolorization values. Despite noticeable differences in their properties, *P. eryngii* SMC and commercial laccase exhibited similar decolorization ability after 3 hr (Fig. 5C), and approximately 93.04% of RBBR was decolorized.

Because of their cost-consuming steps and limited applications, most of the current processes used in treatment of dye wastewater are ineffective and uneconomical [21, 22]. Therefore, development of waste-treatment processes based on laccase appears to be an attractive solution because the products have the potential to degrade dyes...
with diverse chemical structures [23], including synthetic
dyes used in today’s industries [24]. Each 15 µL aliquot of
*P. eryngii* SMC extract and commercial laccase (11 U/mL; Sigma) was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel, including

0.02%, and then stained with 1 mM ABTS. The SMC extract of *P. eryngii* and commercial laccase were stained with coomassie brilliant blue R-250 (Fig. 6). The enzymatic activities were observed on a major band ranging from 55 to 70 kDa and a minor band (34 kDa) by staining with ABTS. The ABTS stained bands of commercial laccase were detected on 53 kDa with comparable intensity of *P. eryngii* SMC samples. The result shows that the *P. eryngii* SMC extract retains a sufficient amount for potential industrial application. In previous reports, two laccase isoenzymes produced by *P. eryngii* were purified to electrophoretic homogeneity and showed molecular masses of 65 and 61 kDa on SDS-PAGE [25]. The pel3 laccase gene was heterogeneously expressed in *Aspergillus niger* and the molecular mass of the recombinant enzyme was estimated as 58 kDa by SDS-PAGE [26]. In addition, a 34 kDa laccase was purified from fruiting bodies of the mushroom *P. eryngii* by ion exchange and gel filtration chromatography [27]. The reported results suggest that molecular masses of laccases of *P. eryngii* can range from 34 to 65 kDa, showing good agreement with our result.

Fungal laccase genes have been overexpressed in different host systems such as yeast and *Aspergillus niger* [20, 25, 28], however, the lack of sufficient enzyme production is the greatest obstacle in commercial application of laccases. In addition, purification of laccase involves several complicated steps that lead to substantial losses of the enzyme [27, 29]. In this study, the SMC that is discarded was found to contain large amounts of lignin-degrading enzymes, and the crude enzymes could be used in decolorization of dyes. Further study is required in order to optimize the purification system for these degradation enzymes in order to make purification inexpensive and suitable for industrial applications and to determine how
the crude enzymes can be stored for extended periods.

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