Enhanced Production of Laccase from *Trametes* sp. by Combination of Various Inducers

Moon Yup Jang, Won Youl Ryu, and Moo Hwan Cho*

School of Chemical Engineering & Technology, Yeungnam University, Kyongsan 712-749, Korea

Abstract In this study, we have attempted to determine the optimum concentration of inducers responsible for efficient laccase production by the white-rot fungus, *Trametes* sp. Variations in laccase activity were investigated with changing concentrations of 2,5-xylidine, syringaldazine, ABTS, and guaiacol. Enhancement of peak laccase activity was achieved via the combination of 2,5-xylidine with ABTS, syringaldazine, or guaiacol, resulting in increases of up to 359, 313, and 340%, respectively, as compared to control values. Among the tested inducers, the addition of 0.1 mM of ABTS coupled with 1.0 mM of 2,5-xylidine in the medium after 24 h of cultivation proved optimal with regard to laccase enzyme production.

Keywords: white-rot fungi, *Trametes* sp., laccase, inducers, 2,5-xylidine, ABTS

INTRODUCTION

Laccase is a member of a small group of enzymes referred to as the large blue copper proteins, or blue copper oxidases. Laccase exhibits an overlapping substrate range, and has been shown to be marrkedly non-specific as to its reducing substrate. Due to its ability to catalyze the oxidation of phenol, laccase has been the focus of an increased amount of attention with regard to its potential biotechnological applications, in fields including delignification [1,2], pulp bleaching [3], contaminated water treatment [4,5], and the detoxification of phenolic compounds [6,7]. Laccase also may be useful in a wide variety of applications in enzyme immunoassays as a marker enzyme [8], in the design of various biosensors [9,10], and in energy transformation systems [11]. However, such determinations would clearly require large quantities of laccase. Fungal sources, at this point, are considered to be the most promising sources for the generation of such large quantities of laccase. In a previous study, we described the selective visualization of lignin peroxidase, manganese peroxidase, and laccase, generated by white-rot fungi on solid media [12]. The simplest for increasing laccase yields is the addition of inducers. Several efforts have been made to find laccase synthesis inducers, using a variety of fungi. 2,5-xylidine has been reported as an effective laccase production inducer when applied to *Pycnoporus cinnabarinus* [13] and *Trametes versicolor* [14]. Laccase production from *Coriolus hirsutus* was augmented via treatment with syringaldazine and guaiacol [15]. Gallic acid and ferulic acid are other example of inducers that enhance laccase production from *Botrytis cinerea* [16] and *Pleurotus sajor-caju* [17]. The genus *Trametes*, a member of the white-rot fungi, is generally considered to be the source of the most efficient known lignin degraders [5,15]. Therefore, the study of the constitutive form of laccase generated by *Trametes* sp. as a component of the ligninolytic system is obviously of substantial significance.

With the aim of further enhancing laccase production via the use of inducers, the present study focused on the synergistic effects of combinations of various inducers on laccase production by *Trametes* sp.

***Corresponding author**
Tel: +82-53-810-2517 Fax: +82-53-814-8790
e-mail: mhcho@yumail.ac.kr

MATERIALS AND METHODS

Chemicals

4-hydroxy-3,5-dimethyoxy-benaldehydeazine (syringaldazine), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2-methoxy-phenol (guaiacol), and 2,5-xylidine were obtained from the Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals used in this study were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

Microorganism

Trametes sp. (KFCC 10941) was taken up as ligninolytic microorganisms. *Trametes* sp. were maintained on 1% (w/v) malt extract agar plates grown at 28°C, and maintained at 4°C until use.

Cultivation Conditions

The basal medium used for laccase-production was the nitrogen-sufficient liquid medium previously described by

Tien and Kirk [18], which used 20 g glucose as the carbon source, and 4 g ammonium tartrate as the nitrogen source, except that in our case, dimethylsuccinate was replaced with 0.1 M succinate buffer (pH 4.5). In the case of a nitrogen-limited medium, the ammonium tartrate concentration was 0.4 g per liter of basal medium. 10 mL of homogenized mycelial pellets were used in the inoculation of a 300 mL flask containing 100 mL of laccase-production medium. After 24 h of cultivation, the syringaldazine, guaiacol, 2,5-xylidine, and ABTS were added as laccase production inducers. Incubation was conducted at 28°C with agitation at 200 rpm. The initial concentration of *Trametes* sp. was adjusted to approximately 0.3 g DCW/L. For the determination of laccase activity, samples of the culture liquid were obtained at daily intervals.

Enzyme Assay

Laccase activity was determined via ABTS oxidation. The assay mixture contained 5 mM ABTS, 0.1 M sodium acetate (pH 5.0), and a suitable amount of enzyme. ABTS oxidation was monitored via determinations of the increase in A_{420} (ε_{420}, 3.6×10^4 $M^{-1} \cdot cm^{-1}$). Enzyme activity was expressed in the following units: 1 U = 1 μmol of ABTS oxidized per minute [19].

RESULTS AND DISCUSSION

Effects of Various Inducers

Low concentrations of several laccases can be constitutively generated on wood and in submerged fungal cultures, but higher concentrations must be produced via the addition of inducers. These are normally the substrates of the enzyme to be produced, or are analogues of natural growth substrates of wild strains of the fungus. Several efforts have been made to identify inducers of laccase synthesis in different fungi [16]. 2,5-xylidine has previously been reported to be the most effective inducer for laccase production in *P. cinnabarinus* [15]. Different types of inducers for *Trametes* sp. have also been examined as stimulating agents for efficient laccase production.

During the growth of the *Trametes* sp., the maximum extracellular laccase contents were detected at the end of the stationary phase. Growth rates and biosynthetic activity along with changing concentrations of 2,5-xylidine were observed in N-sufficient or N-limited medium. The addition of 1.0 mM of 2,5-xylidine to the nitrogen-sufficient culture resulted in the highest degree of laccase activity. Prior to induction, total laccase activity in the nitrogen-sufficient culture was less than 4,500 U/L, but between the 5 to 6th days after induction with 2,5-xylidine, laccase activity peaked, at approximately 12,500 U/L, when the dry cell weight was 7.8 g/L. The most profound laccase activity was observed between the 4 and 6th days (Fig. 1). Another research group reported a similar effect of 2,5-xylidine on laccase production, with increasing laccase activity from 2,000 to 8,000 U/L [20].

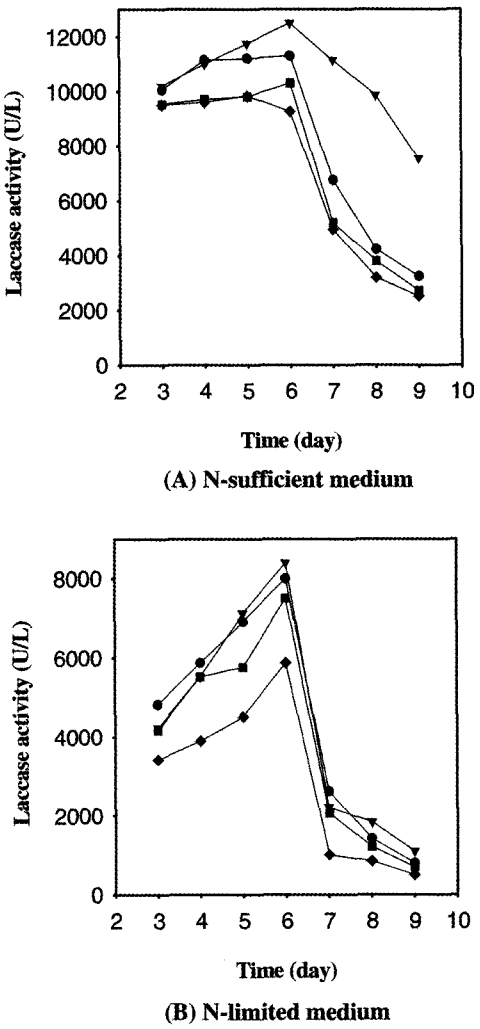

Fig. 1. Variations in laccase activity with changing 2,5-xylidine concentrations during fermentation for (A) N-sufficient or (B) N-limited medium. 2,5-xylidine concentration: ●, 0.5 mM; ▼, 1.0 mM; ■, 1.5 mM; ◆, 3.0 mM.

In our investigations into the effects of the addition of various amounts of inducers including ABTS, guaiacol, and syringaldazine on laccase activity, 0.25 mM of ABTS, 1.0 mM of guaiacol, and 0.1 μM of syringaldazine all were associated with higher laccase activity levels than other concentrations of inducers examined in the nitrogen-sufficient culture, evidencing laccase activity levels of 5,600, 6,200, and 8,880 U/L, respectively (Fig. 2). All of the examined inducers appeared to exert dose-dependent effects on laccase production. These results had been expected, as 2,5-xylidine, ABTS, guaiacol, and syringaldazine are routine substrates for laccase production, or are analogues of natural growth substrates of wild strains of the fungus.

Effects of Combinations of Various Inducers

The enhancement of laccase activity attendant to the

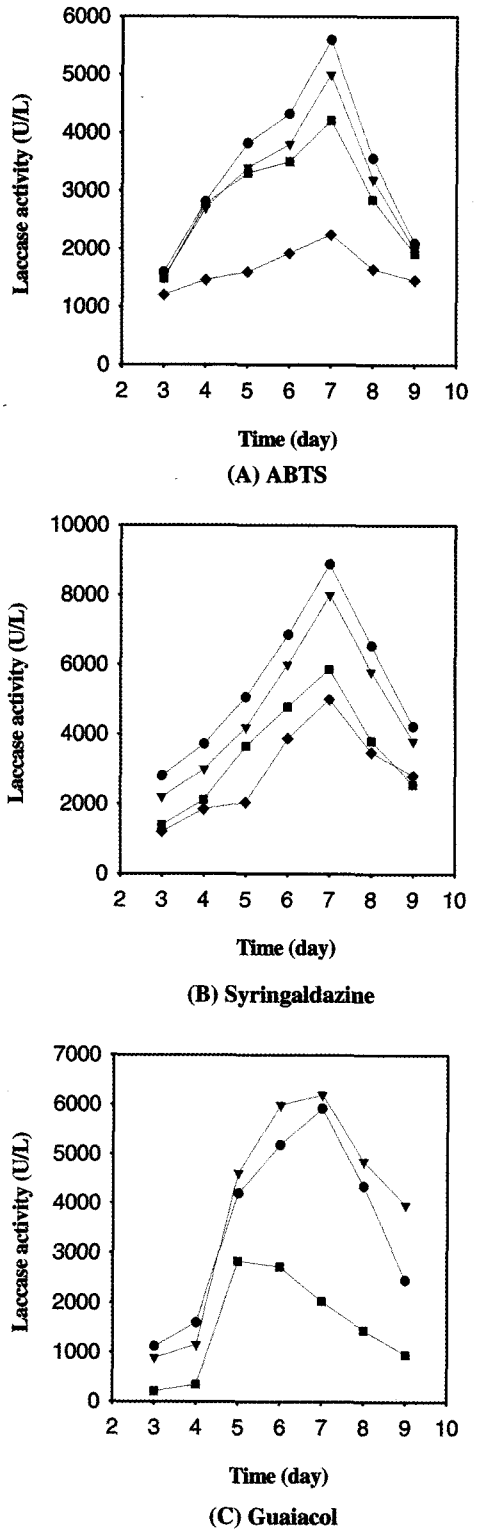

Fig. 2. Effects of ABTS, syringaldazine, and guaiacol on laccase activity during fermentation. (A) ABTS concentration: ▼, 0.1 mM; ●, 0.25 mM; ■, 0.5 mM; ◆, 1.0 mM. (B) Syringaldazine concentration: ▼, 0.05 μM; ●, 0.1 μM; ■, 1.0 μM; ◆, 10 μM. (C) Guaiacol concentration: ●, 0.5 mM; ▼, 1.0 mM; ■, 2.0 mM.

Table 1. Effect of different inducers on laccase biosynthesis

Inducers	Peak laccase activity during fermentation after addition of inducers	
	Activity (U/L)	% of control
Control (without inducer)	4,400	100
ABTS*	5,600	130
Guaiacol*	6,200	141
Syringaldazine*	8,880	202
2,5-Xylidine#	12,500	284
2,5-Xylidine# + Syringaldazine+	13,800	313
2,5-Xylidine# + Guaiacol+	15,000	340
2,5-Xylidine# + ABTS+	15,800	359

ABTS* (0.25 mM), Guaiacol* (1.0 mM), Syringaldazine* (0.1 μM) 2,5-Xylidine# (1.0 mM), Syringaldazine+ (0.1 μM), Guaiacol+ (0.5 mM), ABTS+ (0.1 mM).

addition of 2,5-xylidine coupled with different inducers, such as syringaldazine, guaiacol, and ABTS, was evaluated. The results are summarized in Table 1. The laccase activity in control, with no inducer, was considered for the purposes of the study to be 100%. All of the tested inducers effected increases in the extracellular laccase yield, and this effect peaked on the 6 or 7th day of growth. The addition of ABTS coupled with 2,5-xylidine to the medium after 24 h of incubation proved to be the most efficient laccase biosynthesis inducer, eliciting a 359% increase in laccase activity within the nitrogen-sufficient culture. However, the addition of guaiacol coupled with 2,5-xylidine, and the addition of syringaldazine coupled with 2,5-xylidine, also proved to be efficient inducers, increasing laccase activity levels by 340 and 313%, respectively. Recently, the cooperative effects between inducers on laccase production have been evaluated, showing that mixtures of inducers such as copper, 2,5-xylidine, and phenolic mixtures generated higher laccase activity levels, reaching values of up to 5,500 U/L [21]. Also, Collins and Dobson [22] reported that two inducers of 2,5-xylidine and copper acted synergistically activating laccase gene transcription at a faster rate than evidenced by either when administered alone.

Consequently, among the tested combinations of inducers, the addition of 1.0 mM of 2,5-xylidine coupled with 0.1 mM of ABTS to the medium after 24 h of cultivation was shown to synergistically elicit the highest degree of laccase production.

CONCLUSION

In order to enhance the production of laccase from *Trametes* sp., a variety of inducers, including 2,5-xylidine, ABTS, guaiacol, and syringaldazine were tested. Among these inducers, 2,5-xylidine proved the most effective inducer, effecting an increase of up to 284% in peak laccase activity, as compared to control levels. Also, the coupling of 2,5-xylidine with other inducers resulted in a far more significant enhancement of peak laccase activity than did 2,5-xylidine when administered alone. The op-

timal combination was found to be 1.0 mM 2,5 xylidine coupled with 0.1 mM ABTS, which increased peak laccase activity by up to 359% that of the control.

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