

The effect of various inducers and their combinations with copper on laccase production of *Trametes versicolor* pellets in a repeated-batch process

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Abstract: The aim of this study was to increase laccase production in *Trametes versicolor* ATCC 200801 pellets by using various inducers and their combinations under repeated-batch conditions. Because copper is an effective inducer for laccase production, the effect of Cu on laccase production in this strain was tested first. The optimal Cu concentration for the highest laccase production was 0.5 mM. Following this preliminary study, the effect of other inducers [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), syringaldazine, guaiacol, and 2,5-xylydine] on laccase production was determined. Copper was determined to be the most efficient inducer among the inducers used. Therefore, the synergistic effect of each inducer with Cu on laccase production in this strain was investigated. While the maximum laccase activity was 0.60 U/mL in stock basal medium (SBM) alone, the highest enzyme activities detected were 4.76 and 2.87 U/mL in SBM + 0.05 mM ABTS and SBM + 1 mM 2,5-xylydine, respectively. Maximum laccase activities obtained from the combination of the inducers were 33.61 and 26.49 U/mL in SBM + 0.5 mM Cu + 0.5 mM 2,5-xylydine and SBM + 0.5 mM Cu + 0.05 mM ABTS, respectively. These were the most efficient combinations for laccase production.

Key words: Copper, inducer, laccase, repeated-batch process, *Trametes versicolor*

1. Introduction

Laccases (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductases) are industrially important multicopper-containing enzymes. Although laccases are produced by some higher plants, insects, bacteria, and fungi, these enzymes are mainly abundant in white rot fungi (Gochev and Krastanov, 2007; Shraddha et al., 2011). These fungi, including *Trametes*, *Pleurotus*, *Coriolopsis*, and other genera, produce laccase enzymes at varying rates (Tapia-Tussell et al., 2011). *Trametes* is one of the most effective laccase-producing genera (Jang et al., 2002; Rosales et al., 2007). Fungal laccases are generally extracellular and they catalyze the oxidation of a wide range of compounds, such as mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines, ascorbate, and nonphenolic compounds (Thurston, 1994; Couto et al., 2002; Baldrian, 2006; Patel and Gupte, 2016). The nonnecessity of any extraction process for obtaining these enzymes and their low substrate specificities make laccases suitable for many industrial applications, such as pulp delignification and biobleaching, textile dye decolorization, wastewater treatment, fruit juice processing, and construction of biosensors and biofuel cells (Minussi et al., 2002; Giardina et al., 2010; Durán et

al., 2014; Yeşilada et al., 2014). Due to their broad industrial and environmental uses, these enzymes have gained increasing attention. Laccase production can be stimulated by various inductive substances, including metal ions, aromatic or phenolic compounds, alcohol, and detergents (Collins and Dobson, 1997; Birhanli and Yesilada, 2006; Tychanowicz et al., 2006; Bettin et al., 2014). Furthermore, inducer concentration, fermentation types, growth media, and incubation conditions have an important effect on laccase production and they are specific to species and strains (Boran and Yesilada, 2011). Many researchers have investigated the effects of various fermentation processes, cultivation conditions, laccase-producing organisms, and inducers on laccase production (Minussi et al., 2007; Elisashvili and Kachlishvili, 2009; Birhanli and Yesilada, 2010; Dhillon et al., 2012; Birhanli and Yeşilada, 2013; Manavalan et al., 2013). Some of these researchers used immobilized fungal cells instead of dispersed cells for laccase production in their studies, as immobilized cells make it easy to separate cells from liquid culture medium (Dominguez et al., 2007; Neifar et al., 2009). However, immobilization processes have some disadvantages, such as the high cost, immobilization matrix damage, and oxygen supply problems during cultivation. It is possible

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to overcome these problems by using fungal pellets, which can also be a self-immobilization system (Wang et al., 2005; Birhanli and Yesilada, 2006).

In the present work, *Trametes versicolor* (*T. versicolor*) ATCC 200801 was chosen as an excellent laccase-producing white rot fungus according to our previous studies (Birhanli and Yesilada, 2006; Boran and Yesilada, 2011; Birhanli and Yeşilada, 2013). Since fungal pellets can be used repeatedly for high amounts of laccase production, the repeated-batch method was used as the fermentation process. There are limited studies available on laccase production using the repeated-batch method (Jang et al., 2002; Birhanli and Yesilada, 2010; Baccar et al., 2011; Casas et al., 2013; Wang et al., 2013). The aim of our study was to understand the effect of inducers and inducer combinations on laccase production in *T. versicolor* ATCC 200801 using the repeated-batch method. Therefore, in the first part of the study, *T. versicolor* ATCC 200801 pellets were incubated in SBM (control) without any inducers, and the effects of different concentrations of Cu, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), syringaldazine, guaiacol, and 2,5-xylidine on laccase production in the repeated-batch method in this fungus were investigated. Moreover, combinations of these inducers with Cu (0.5 mM) were also tested to determine their synergistic effects on laccase production. Some studies have also tested inductive substances and/or their combinations for the same purpose by using various white rot fungi under different cultivation processes (Koroljova-Skorobogačko et al., 1998; D'Souza et al., 2006; Cordi et al., 2007; Schüchel et al., 2011; Aty and Mostafa, 2013). However, this is the first study on the effect of various inducers and also their combinations on laccase production in *T. versicolor* ATCC 200801 under repeated-batch cultivation conditions.

2. Materials and methods

2.1. Chemicals

Copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Sabouraud dextrose agar (SDA), and Sabouraud dextrose broth (SDB) were purchased from Merck (Darmstadt, Germany). ABTS, syringaldazine, 2,5-xylidine, SDS protein standards, and laccase from *T. versicolor* (commercial laccase, 26.8 U/mg) were obtained from Sigma-Aldrich Chemical Co. (Steinheim, Germany). Guaiacol was procured from Acros Organics (Morris Plains, NJ, USA).

2.2. Stock culture maintenance and storage

T. versicolor ATCC 200801 growing on *Cupressus* sp. was originally collected from Adana, Turkey. The stock culture of this fungus is in the biotechnology laboratory of the Arts and Science Faculty at İnönü University, Malatya, Turkey. The fungus was first subcultured at 30 °C every 2–3 weeks on SDA plates in a static incubator and then maintained at 4 °C in a refrigerator.

2.3. Preparation of inoculum and fungal pellets

A portion of fungal mycelia was transferred from stock SDA plates to slant SDA and incubated at 30 °C for a week. After 1 week, sterilized distilled water was pipetted on the slant SDA culture and mycelial suspension was prepared by rubbing the mycelia with a sterilized loop. Then 5 mL of suspension (dry weight: 0.028 g/5 mL) was inoculated into a 250-mL Erlenmeyer flask containing 100 mL of sterile SDB and incubated at 30 °C and 150 rpm in a shaking incubator for 5 days. After incubation, the preculture was homogenized under aseptic conditions and 7 mL of homogenates (dry weight: 0.039 g/7 mL) were inoculated into 600 mL of fresh SDB media in 1000-mL Erlenmeyer flasks. After that, the homogenates were incubated for fungal pellet production under the same conditions as mentioned above for 5 days. After the formation of spherical pellets (about 2.0–2.5 mm in diameter), they were harvested by filtrating with aseptic plastic filters and then 30 g wet (0.70 g dry weight) of fungal pellets were transferred separately to 50 mL of SBM only and SBM with inducers. After that, the pellets were used for laccase production (Birhanli and Yesilada, 2010; Yesilada et al., 2014).

2.4. Laccase production via the repeated-batch process

First, the harvested fungal pellets were incubated in 50 mL of SBM with 0.25–5 mM of Cu. SBM and SBM with 0.5 mM Cu containing separately 0.025–1 mM ABTS, 0.025–0.5 mM syringaldazine, 0.05–1.5 mM guaiacol, and 0.05–1.5 mM 2,5-xylidine were used as the enzyme production media in repeated-batch fermentation. After this preliminary process, the fungal pellets were transferred to these media and repeated-batch operations with different cycle times were performed without removing the pellets. SBM used for the studies had the following composition (g/L): KH_2PO_4 0.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05; $\text{NH}_4\text{H}_2\text{PO}_4$ 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.035, glucose 2, yeast extract 1. All the experiments were performed under the optimum conditions described in our previous study (Birhanli and Yesilada, 2010).

2.5. Laccase activity assay

In order to detect the effects of different inducers and their concentrations on laccase production in *T. versicolor* ATCC 200801 pellets, the culture fluids were filtrated every 24 h and their laccase activities were determined. Laccase activity was detected spectrophotometrically (Shimadzu-UV-1601, UV/Visible spectrophotometer; Shimadzu, Kyoto, Japan) by monitoring the oxidation of ABTS at 420 nm ($\epsilon_{420} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) for 1 min at 30 °C. The reaction mixture contained 833 μL of sodium acetate buffer (100 mM, pH 5.0), 100 μL of ABTS (0.5 mM), and a suitable amount of crude laccase enzyme. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min at 30 °C (Murugesan et al., 2007; Birhanli and Yeşilada, 2013).

2.6. Statistical analysis

The raw absorbance data obtained from spectrophotometric measurements were evaluated statistically with SPSS 15.0 for Windows. After this statistical evaluation, the laccase enzyme activities in the culture filtrates were expressed as U/mL. All laccase activity values are the means of at least 3 replicates, with standard deviation of the mean shown as \pm values.

2.7. Determination of molecular weight of laccase by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the culture filtrate obtained from SBM + 0.5 mM Cu + 0.05 mM ABTS was performed on 10% separating and 4% stacking gels. Protein standard mixture containing 8 different protein standard markers with molecular masses ranging from 14.2 to 205 kDa (Sigma-M2789) [α -lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa),

albumin (BSA) (66 kDa), phosphorylase-B (97.4 kDa), β -galactosidase (116 kDa), and myosin (205 kDa)] was used for determination of molecular weight of *T. versicolor* ATCC 200801 laccase. In addition to *T. versicolor* ATCC 200801 laccase, commercial laccase was also used as a positive control. Accordingly, the protein standard mixture, commercial laccase, and *T. versicolor* culture filtrate with a high amount of laccase were migrated in a 40 mA/gel static electric current. After electrophoresis, protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Figure 1A). In addition to SDS-PAGE, native PAGE was also performed under nondenaturing conditions. After electrophoresis, a native polyacrylamide gel was incubated in ABTS solution to visualize laccase activity. Commercial *T. versicolor* laccase (BioChemika Fluka-53739, Chemika, Buchs, Switzerland) was also used in native PAGE as a positive control (Figure 1B).

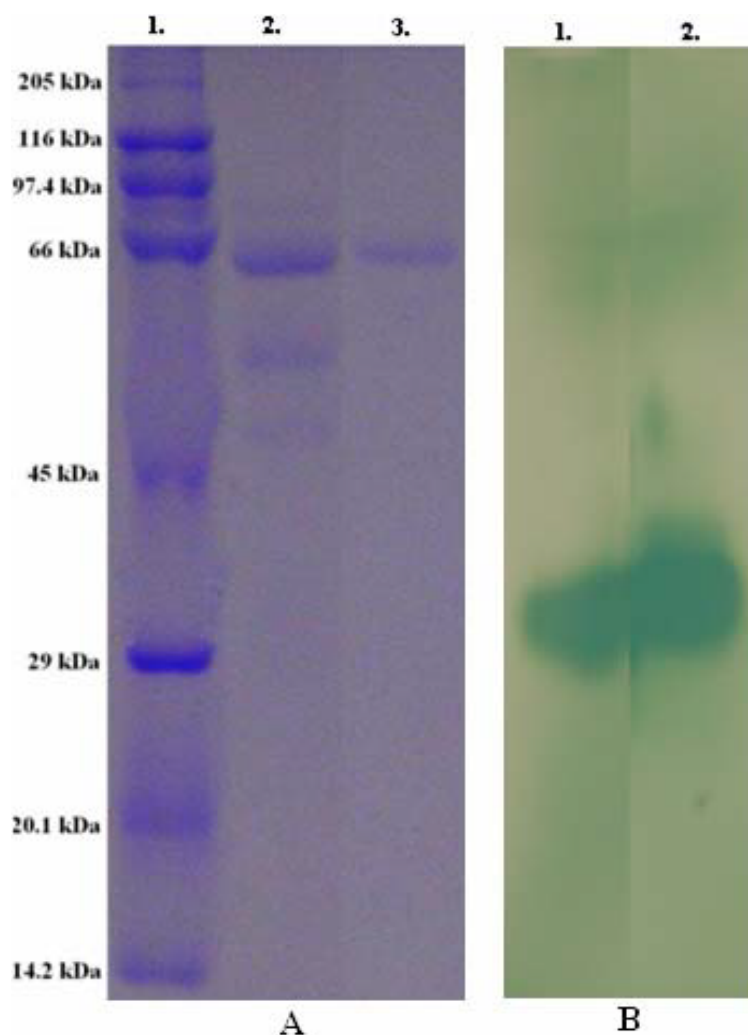


Figure 1. SDS-PAGE (A) and native PAGE (B) of commercial laccase and culture filtrate of *T. versicolor* ATCC 200801 pellets obtained from Y + 0.05 mM ABTS. Lane 1, molecular weight markers; lane 2, commercial laccase; lane 3, culture filtrate in SDS-PAGE. Lane 1, commercial laccase; lane 2, culture filtrate in native PAGE (Y: SBM + 0.5 mM Cu).

3. Results

3.1. Effect of different concentrations of Cu on laccase production in *T. versicolor* ATCC 200801 pellets

T. versicolor ATCC 200801 pellets were incubated in SBM alone (control) and SBM with Cu (0.25–5 mM) to determine the optimum concentration of Cu for high laccase enzyme production. The pellets were incubated in these media for 11 days under repeated-batch conditions, and their laccase activities were measured spectrophotometrically every 24 h. When the pellets were incubated in SBM with 0.25–2 mM Cu, the laccase activities rapidly increased in comparison to the control. The most effective Cu concentration for laccase production was 0.5 mM. Conversely, 5 mM Cu showed an inhibitory effect and therefore the enzyme activity in this medium was even lower than the control. Accordingly, the highest laccase activities were 0.60, 10.25, and 0.19 U/mL in SBM, SBM + 0.5 mM Cu, and SBM + 5 mM Cu, respectively (Table 1).

3.2. Effect of different concentrations of ABTS and ABTS with Cu on laccase production in *T. versicolor* ATCC 200801 pellets

The pellets were incubated in SBM with 0.025–1 mM ABTS and SBM without ABTS in order to study the effect of ABTS on laccase production. The highest laccase activities were observed in 0.025 and 0.05 mM ABTS (3.64 and 4.76 U/mL on day 4, respectively). The other ABTS concentrations did not show any significant effect on laccase production in this strain (Figure 2A).

Because Cu was detected as the best inducer and 0.5 mM Cu was determined as the most effective concentration for

laccase production, SBM with ABTS + 0.5 mM Cu was also used as the culture medium for testing the synergistic effect of two inducers on laccase production in the pellets under repeated-batch conditions. All Cu + ABTS concentrations induced laccase production; the best was 0.5 mM Cu + 0.05 mM ABTS (Figure 2B). While the laccase activities were 5.78 U/mL and 4.76 U/mL in SBM + 0.5 mM Cu and SBM + 0.05 mM ABTS media on day 4, the laccase activity value on the same day was 26.49 U/mL in SBM + 0.5 mM Cu + 0.05 mM ABTS medium. This shows that laccase production could be significantly induced by using Cu + ABTS as a synergistic inducer. Laccase activities gradually increased in all media until day 4. While the maximum enzyme activities in SBM + 0.025 mM ABTS and SBM + 0.05 mM ABTS were 3.64 and 4.76 U/mL (Figure 2A), the highest laccase activities in SBM + 0.5 mM Cu + 0.025 mM ABTS and SBM + 0.5 mM Cu + 0.05 mM ABTS were 20.87 and 26.49 U/mL, respectively (Figure 2B). The laccase activities in SBM alone and SBM + 0.5 mM Cu on the same day were 0.54 and 5.78 U/mL, respectively (Figure 2B).

3.3. Effect of different concentrations of syringaldazine and syringaldazine with Cu on laccase production in *T. versicolor* ATCC 200801 pellets

In order to determine the effect of syringaldazine alone on laccase production, various concentrations (0.025, 0.05, 0.1, and 0.5 mM) of syringaldazine were added to SBM. The same concentrations of syringaldazine were also supplemented separately to SBM + 0.5 mM Cu for detecting the induction capacity of syringaldazine with Cu on laccase production. During the 5-day incubation, there was no induction of laccase production in SBM with several amounts of syringaldazine. Accordingly, the

Table 1. Laccase production in *T. versicolor* ATCC 200801 pellets in SBM alone or SBM with various concentrations of Cu (U/mL).

Time (days)	SBM	SBM + 0.25 mM Cu	SBM + 0.5 mM Cu	SBM + 1 mM Cu	SBM + 2 mM Cu	SBM + 5 mM Cu
1	0.42 ± 0.01	1.44 ± 0.29	3.60 ± 0.20	2.49 ± 0.22	0.58 ± 0.04	0.13 ± 0.01
2	0.45 ± 0.01	2.11 ± 0.29	4.13 ± 0.12	2.85 ± 0.15	1.07 ± 0.03	0.19 ± 0.02
3	0.51 ± 0.01	2.63 ± 0.18	5.19 ± 0.45	3.46 ± 0.36	1.67 ± 0.16	0.18 ± 0.01
4	0.54 ± 0.03	3.07 ± 0.31	5.78 ± 0.35	3.89 ± 0.09	2.07 ± 0.15	0.14 ± 0.02
5	0.60 ± 0.04	3.54 ± 0.41	6.33 ± 0.10	4.31 ± 0.34	1.50 ± 0.25	0.06 ± 0.02
6	0.53 ± 0.01	4.48 ± 0.30	10.25 ± 0.30	4.98 ± 0.40	0.60 ± 0.01	0.03 ± 0.01
7	0.48 ± 0.03	3.64 ± 0.26	9.53 ± 0.48	4.67 ± 0.37	0.23 ± 0.02	0.02 ± 0.00
8	0.43 ± 0.03	2.38 ± 0.17	4.75 ± 0.63	1.75 ± 0.13	0.15 ± 0.01	0.01 ± 0.01
9	0.30 ± 0.06	0.65 ± 0.07	2.93 ± 0.33	0.94 ± 0.06	0.07 ± 0.01	0.01 ± 0.00
10	0.19 ± 0.03	0.44 ± 0.08	1.54 ± 0.15	0.64 ± 0.06	0.06 ± 0.01	0.01 ± 0.00
11	0.14 ± 0.01	0.34 ± 0.03	0.69 ± 0.12	0.48 ± 0.07	0.04 ± 0.00	0.01 ± 0.00

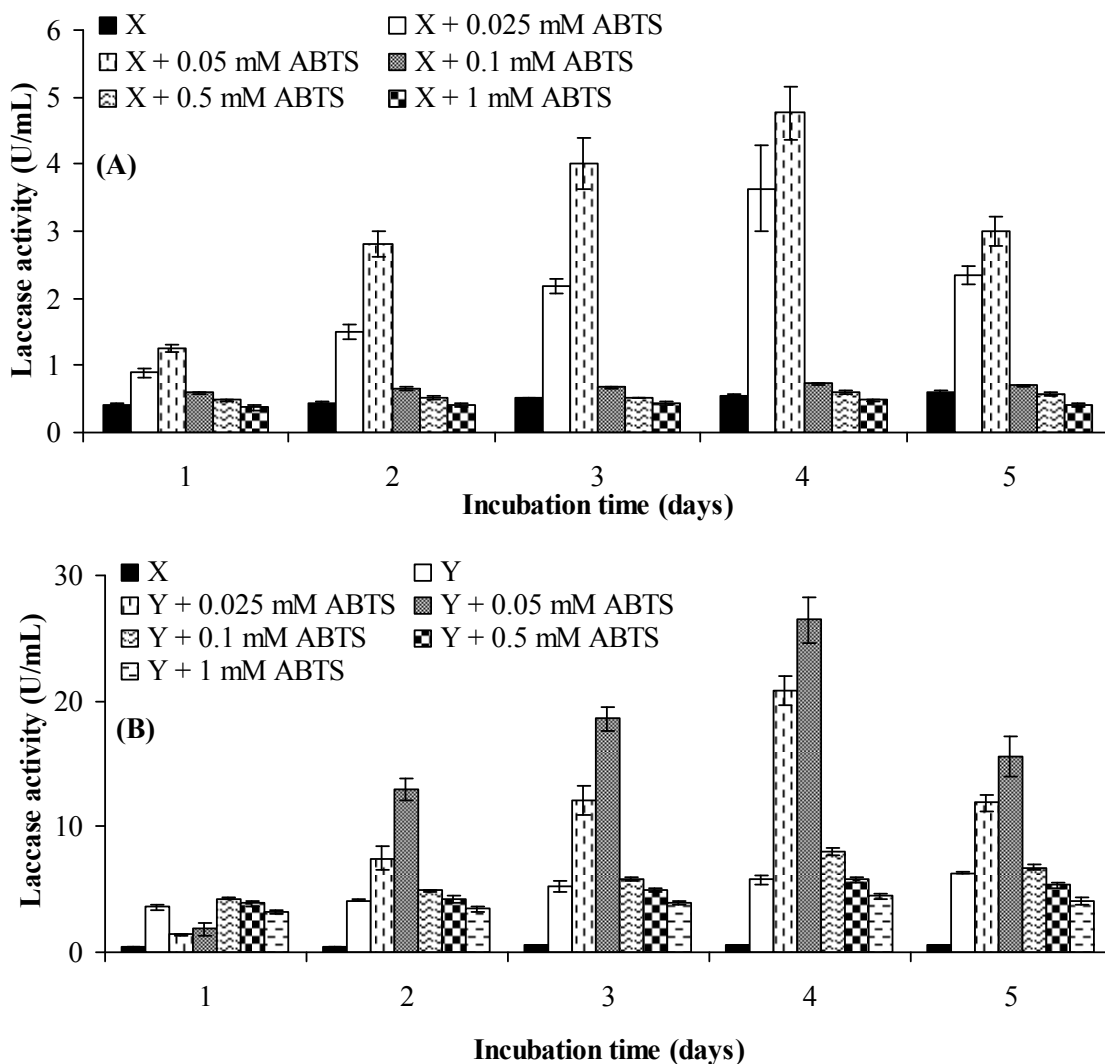


Figure 2. Laccase production in *T. versicolor* ATCC 200801 pellets in SBM (A) and SBM + 0.5 mM Cu (B) with various concentrations of ABTS (X: SBM, Y: SBM + 0.5 mM Cu).

highest enzyme activities were 0.60, 0.31, and 0.26 U/mL in SBM, SBM + 0.1 mM syringaldazine, and SBM + 0.05 mM syringaldazine, respectively (Figure 3A). However, all tested concentrations of syringaldazine with 0.5 mM Cu induced laccase production and the maximum laccase activities reached 22.23 and 16.41 U/mL in SBM + 0.5 mM Cu + 0.1 mM syringaldazine and SBM + 0.5 mM Cu + 0.05 mM syringaldazine, respectively (Figure 3B).

3.4. Effect of different concentrations of guaiacol and guaiacol with Cu on laccase production in *T. versicolor* ATCC 200801 pellets

The other tested inducer, guaiacol, was added separately to SBM alone and SBM + 0.5 mM Cu at final concentrations of 0.05, 0.1, 0.5, 1, and 1.5 mM. To investigate the induction potential of guaiacol alone and its synergistic effect with Cu, the pellets were cultivated in these media for 10 days

and the laccase activities were detected every 24 h. SBM with guaiacol media induced more laccase production than SBM without an inducer. SBM with Cu + guaiacol media repressed the enzyme production compared to SBM with only Cu. The maximum laccase activities, detected on day 6, were 2.02 U/mL and 1.74 U/mL in SBM + 1 mM guaiacol and SBM + 0.5 mM guaiacol media, respectively. These enzyme activity values were 3.4- and 2.9-fold higher than the highest laccase activity of the control (0.60 U/mL) (Figure 4A). The best enzyme activity values in SBM + 0.5 mM Cu with different amounts of guaiacol were 5.71 and 4.68 U/mL in SBM + 0.5 mM Cu + 1 mM guaiacol and SBM + 0.5 mM Cu + 0.5 mM guaiacol, respectively. Both of these enzyme activity values were lower than the maximum laccase activity in SBM + 0.5 mM Cu (10.25 U/mL) (Figure 4B).

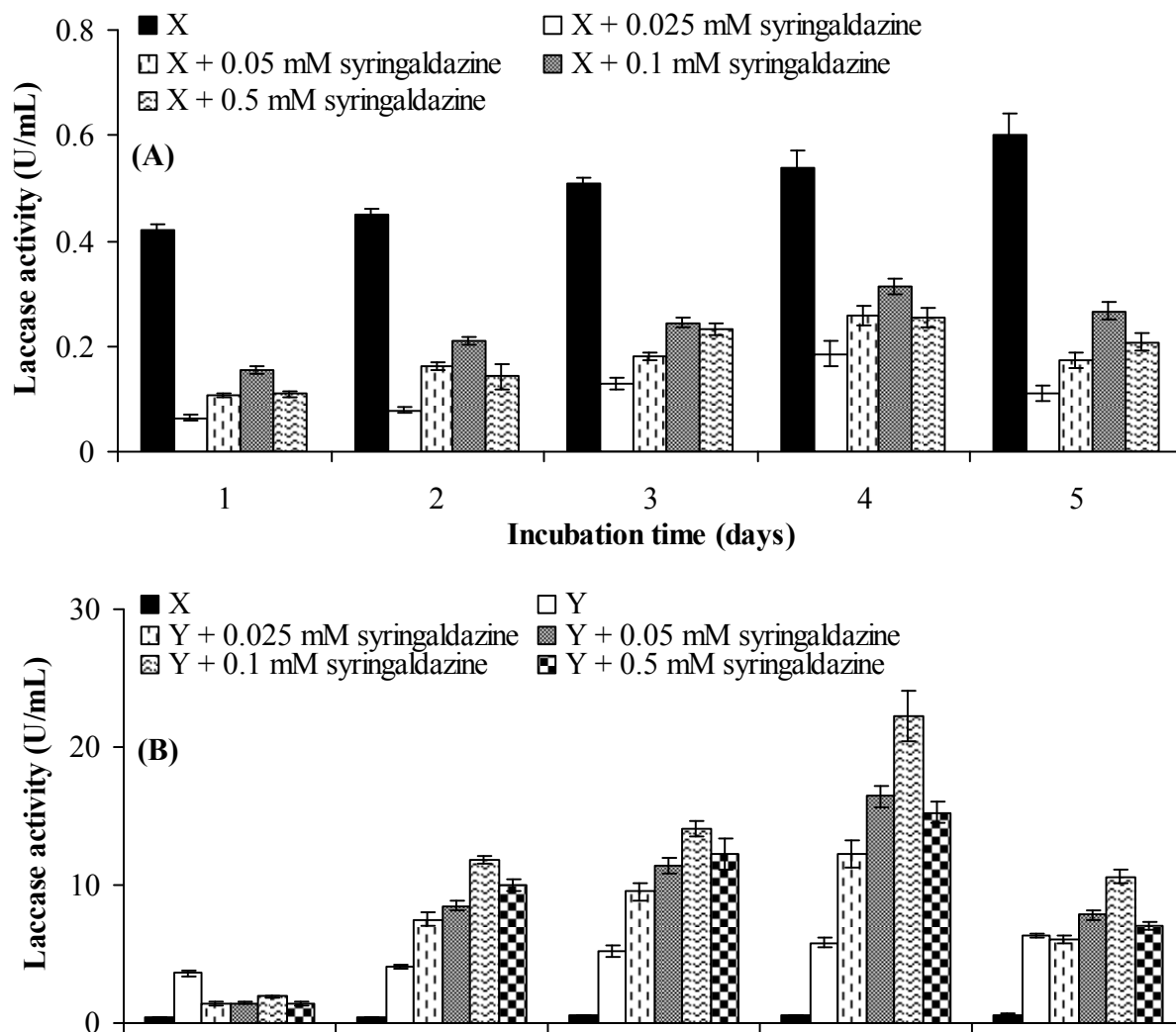


Figure 3. Laccase production in *T. versicolor* ATCC 200801 pellets in SBM (A) and SBM + 0.5 mM Cu (B) with various concentrations of syringaldazine (X: SBM, Y: SBM + 0.5 mM Cu).

3.5. Effect of different concentrations of 2,5-xylydine and 2,5-xylydine with Cu on laccase production in *T. versicolor* ATCC 200801 pellets

2,5-Xylydine was added to SBM alone and SBM + 0.5 mM Cu at final concentrations of 0.05, 0.1, 0.5, 1, and 1.5 mM, respectively. The fungal pellets were incubated in these media for 10 days to analyze the induction capacity of 2,5-xylydine with Cu and without Cu. The presence of 2,5-xylydine in both SBM alone and SBM + 0.5 mM Cu media led to an increase in laccase production. The maximum laccase activity values were 2.87 and 1.17 U/mL in SBM containing 1 and 1.5 mM 2,5-xylydine media, respectively (Figure 5A). Enzyme production in the pellets was enhanced considerably by the synergistic effect of 2,5-xylydine and Cu. The highest amounts of laccase enzyme were obtained from SBM + 0.5 mM Cu including

0.5 mM (33.61 U/mL), 1 mM (31.27 U/mL), and 0.1 mM 2,5-xylydine (29.58 U/mL) as compared with the other inducers used in this study. These laccase activities were about 56-, 52-, and 49-fold higher than the maximum laccase activity of the control (0.60 U/mL), respectively (Figure 5B).

3.6. Molecular weight of laccase

The molecular weight of the crude laccase of the culture filtrate obtained from SBM + 0.5 mM Cu + 0.05 mM ABTS was calculated by SDS-PAGE. Additionally, the molecular weights of crude laccase and also commercial laccase were assayed by this technique for comparison with the crude and purified forms of *T. versicolor* (commercial) laccase. Crude *T. versicolor* ATCC 200801 laccase had an extensive single-protein band and the molecular weight of this laccase was determined as about 65 kDa (Figure 1A).

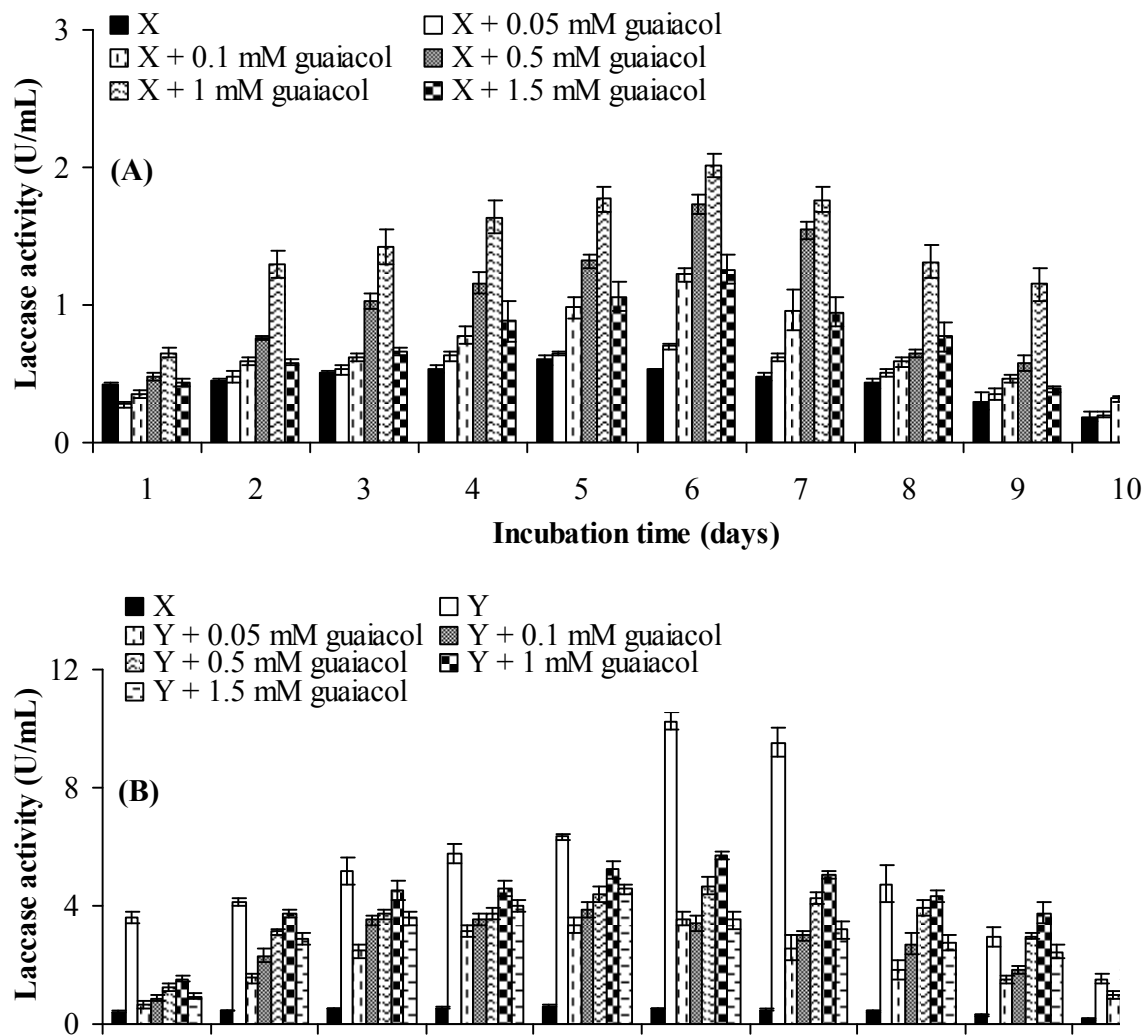


Figure 4. Laccase production in *T. versicolor* ATCC 200801 pellets in SBM (A) and SBM + 0.5 mM Cu (B) with various concentrations of guaiacol (X: SBM, Y: SBM + 0.5 mM Cu).

Native PAGE also revealed extensive laccase activity for the filtrate used (Figure 1B).

4. Discussion

The transcriptional regulation of the laccase gene is affected by several different factors, such as nutrient levels and culture conditions (Giardina et al., 2010). Laccase gene transcription is regulated by metal ions and various inducers (Giardina et al., 2010; Piscitelli et al., 2011). Since Cu atoms serve as cofactors in the catalytic center of laccases and also regulate them at the level of gene transcription, Cu has been used for inducing laccase production in many studies (Majeau et al., 2010). It has been reported that the laccase gene is transcriptionally regulated by Cu in *T. versicolor* (Collins and Dobson, 1997), *P. ostreatus* (Faraco et al., 2003), *P. sajor-caju* (Soden and Dobson, 2001), *T.*

pubescens (Galhaup et al., 2002), and *T. velutina* (Yang et al., 2013). In addition to its effect on laccase synthesis at the transcriptional level, Cu serves as a protease inhibitor, thus reducing laccase degradation (Palmieri et al., 2001). While Cu ions in low concentrations (millimolar ranges) are necessary for laccase production, Cu ions in high concentrations may be toxic to fungal growth and thus may inhibit laccase production (Zeng et al., 2011). Depending on fungal species and even strains, the concentrations of Cu causing induction or inhibition on laccase production may vary. Kumar et al. (2011) reported that copper sulfate at 1 mM concentration enhanced laccase production in *F. solani* (80%), *P. ostreatus* (60%), and *A. bisporus* (54%). The highest laccase production was obtained from *P. ostreatus* (910 U/L) on day 9 at 1 mM copper sulfate. Galhaup and Haltrich (2001) determined the laccase production of

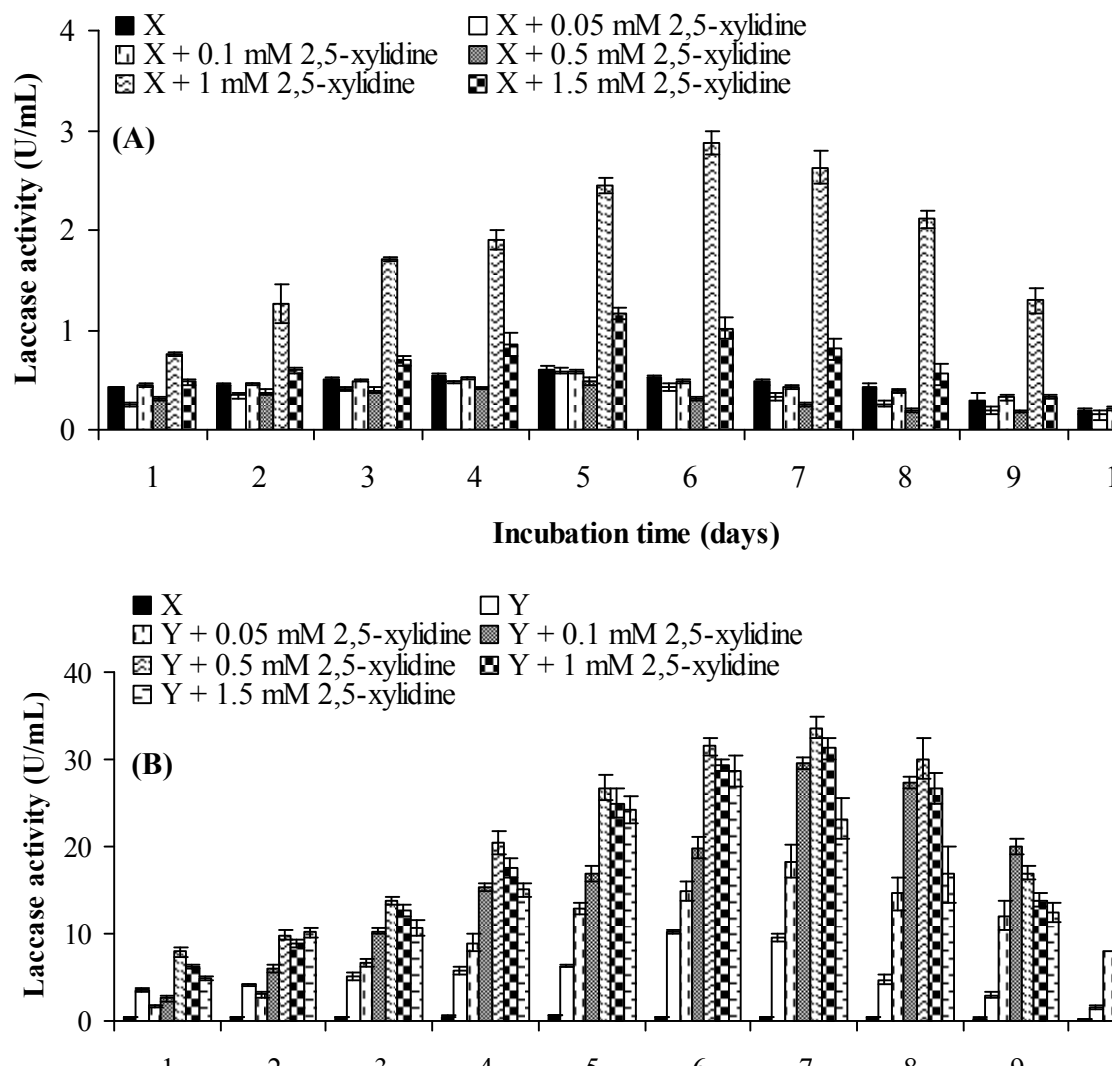


Figure 5. Laccase production in *T. versicolor* ATCC 200801 pellets in SBM (A) and SBM + 0.5 mM Cu (B) with various concentrations of 2,5-xylydine (X: SBM, Y: SBM + 0.5 mM Cu).

different white rot species and even *T. versicolor* MB 52 and MB 54 strains were stimulated at different rates by 1 mM Cu^{2+} . In addition, they also studied the effect of various concentrations of Cu (0.1–2.0 mM) on laccase production in *T. pubescens* MB 89. While the low concentrations of Cu (0.1–1.0 mM) did not cause any inhibition of growth, higher concentrations (1.5–2.0 mM) partially inhibited fungal growth. In another study 2, 3.5, and 5 mM Cu^{2+} were supplemented to culture medium with barley bran and the maximum laccase activity of *T. versicolor* CBS100.29 was 8277 U/L in culture medium with 3.5 mM Cu^{2+} (Lorenzo et al., 2006). Hence, *T. versicolor* ATCC 200801 pellets were incubated in SBM with different amounts of Cu (at final concentrations of 0.25–5 mM) for 11 days in this study. The maximum laccase activity (10.25 U/mL) was obtained from SBM + 0.5 mM Cu on day 6 of incubation, and this

activity value was 17-fold higher than the highest laccase activity in SBM. Therefore, in order to determine the effect of the combination of Cu with the inducers on laccase production, different inducers at various concentrations were supplemented to SBM + 0.5 mM Cu medium, and then the pellets were incubated in these media.

Only a limited number of studies have tested the effect of nonphenolic heterocyclic compound ABTS on laccase production. Hou et al. (2004) used different inducers, such as ABTS, veratryl alcohol, guaiacol, 2,5-xylydine, ferulic acid, and tyrosine, for improving laccase production in *P. ostreatus* strain 32. ABTS displayed the most significant effect amongst all tested inducers; laccase activity was increased about five-fold compared to the control. In a study conducted by Dhillon et al. (2012), several inducers, including ABTS, were used in order to increase laccase

Table 2. Effect of different inducers and their combinations with Cu on laccase production.

Media	Day maximum laccase activity was obtained	Maximum laccase activity (U/mL)
X	5	0.60 ± 0.04
Y	6	10.25 ± 0.30
X + 0.05 mM ABTS	4	4.76 ± 0.40
X + 0.1 mM syringaldazine	4	0.31 ± 0.01
X + 1 mM guaiacol	6	2.02 ± 0.09
X + 1 mM 2,5-xylydine	6	2.87 ± 0.12
Y + 0.05 mM ABTS	4	26.49 ± 1.83
Y + 0.1 mM syringaldazine	4	22.23 ± 1.85
Y + 1 mM guaiacol	6	5.71 ± 0.11
Y + 0.5 mM 2,5-xylydine	7	33.61 ± 1.22

(X: SBM, Y: SBM + 0.5 mM Cu)

production in *T. versicolor* ATCC 20869 under solid-state fermentation conditions in Erlenmeyer flasks and plastic tray bioreactors. After 12 days of incubation, while the laccase activities of control and 2 mM ABTS-supplemented media in flasks were 2007 and 5284 U/g, the activities obtained from the control and 2 mM ABTS-supplemented media in plastic trays were 2956 and 6445 U/g, respectively. Aty and Mostafa (2013) reported that copper sulfate (2 mM) is a more effective inducer than guaiacol (1 mM) and ABTS (1 mM) on stationary cultures of the marine-derived fungus *A. alternata*. In contrast to the work mentioned above, 0.6 mM ABTS stimulated more laccase production in *T. viride* NFCCI 2745 than Cu sulfate (0–2 mM) and 0.6 mM guaiacol (Divya et al., 2013). In this study, ABTS induced laccase production and 4.76 U/mL laccase activity was detected in SBM. On the other hand, 0.5 mM Cu + 0.05 mM ABTS showed a strong synergistic effect on laccase production in *T. versicolor* ATCC 200801 pellets under repeated-batch conditions and 26.49 U/mL laccase activity was obtained. It was 44-fold higher than the laccase activity of the control.

Many phenolic or aromatic compounds, such as syringaldazine, guaiacol, and 2,5-xylydine, have also been used to stimulate production of the ligninolytic enzyme laccase (Couto et al., 2002; Majeau et al., 2010). Syringaldazine was used in some studies for stimulating laccase production in white rot fungi. According to our literature survey, various studies have achieved different results regarding the effect of syringaldazine on laccase production, depending on factors such as inducer concentration, the content of the cultivation medium, and the types of fermentation and organisms used. Koroljova-

Skorobogat'ko et al. (1998) reported that syringaldazine is a major contributor to laccase production in *C. hirsutus*, and Rosales et al. (2007) stated that 0.11 µmol/L syringaldazine in ground orange peelings culture medium stimulated laccase activity in *T. hirsuta*, in comparison to control cultures, but 1 mM Cu sulfate + 0.11 µmol/L syringaldazine showed no significant increase in laccase activity. On the other hand, 0.11 µmol/L did not lead to a noticeable increase in laccase activity in *C. hirsutus*, *C. fulvocinerea*, or *C. maxima* (Gorbatova et al., 2006). In another study, 0.21 µmol/L syringaldazine caused a decrease in laccase activity in *T. versicolor* 1666 strain cultivated under submerged conditions (Shakhova et al., 2011). In the present study, syringaldazine was also tested alone or in combination with Cu for enhancing laccase production. For this purpose, the pellets were first incubated for 5 days in SBM with various concentrations (0.025–0.5 mM) of syringaldazine, but no laccase induction was observed. In contrast, when the pellets were incubated in SBM with 0.5 mM Cu + different concentrations of syringaldazine, significant levels of laccase production were induced. Laccase activity increased considerably with the synergistic effect of 0.5 mM Cu + 0.1 mM syringaldazine in particular, and it reached 22.23 U/mL on day 4. This maximum laccase activity value was almost 37 times higher than the highest laccase activity of the control.

Guaiacol was also reported as an effective inducer for laccase production. Guaiacol of 1 mM induced laccase production in *T. versicolor* ATCC 20869 (Lee et al., 1999). Such an inductive effect of 1 mM guaiacol on *Trametes pubescens* MB 89 (Galhaup and Haltrich 2001), *P. ostreatus* strain 32 (Hou et al., 2004), and the marine basidiomycetous

fungus NIOCC # 2a (D'Souza et al., 2006) was also reported. Yang et al. (2013) stated that metal ions such as Cu^{2+} (0.5 and 1 mM) and aromatic compounds such as guaiacol (1 mM) could stimulate laccase gene transcription and laccase synthesis in *T. velutina* 5930. However, the same concentration of guaiacol did not show a stimulatory effect on laccase production in *P. cinnabarinus* (Eggert et al., 1996) and *T. hirsuta* (Bakkiyaraj et al., 2013). This might be due to the differences of culture conditions and fungal species tested. In our study, all the concentrations of guaiacol used showed a stimulatory effect on laccase production in *T. versicolor* ATCC 200801 pellets incubated under repeated-batch conditions, and the maximum laccase activity obtained from SBM + 1 mM guaiacol was about 3.4 times greater than the highest laccase activity of the control. On the other hand, 0.5 mM Cu + guaiacol did not stimulate laccase production compared to SBM + 0.5 mM, but the activities detected in 0.5 mM Cu + guaiacol culture media were much higher compared to SBM.

A high amount of laccase enzyme could be produced by using a suitable inducer or combination thereof, and thus this enzyme can be produced on industrial scale (Majeau et al., 2010; Patrick et al., 2011). The commonly used inducer 2,5-xylydine was also used to test its inducing effect on laccase production in *T. versicolor* ATCC 200801 under repeated-batch conditions. Although 0.05–0.5 mM 2,5-xylydine did not improve enzyme production, 1 and 1.5 mM 2,5-xylydine stimulated more laccase production than the control. Patrick et al. (2011) reported the toxicity of high concentrations of xylydine. Maximum laccase activity was obtained from SBM + 1 mM 2,5-xylydine as 2.87 U/mL, and this activity was almost 4.8-fold higher than the maximum laccase activity in SBM. This result is in accordance with the findings reported by Manavalan et al. (2013) that laccase production in *G. lucidum* increased to 2.36 U/mL through the addition of 1 mM xylydine. Similarly, various concentrations of 2,5-xylydine led to an effective stimulation of laccase production in various organisms, such as *T. rubrum* LKY-7 (Jung et al., 2002), *T. versicolor*, *T. villosa*, *B. cinerea* (Minussi et al., 2007), and *P. sajor-caju* (Patrick et al., 2011). According to Lee et al. (1999), approximately 1 U/mL of laccase activity was obtained by *T. versicolor* ATCC 200869 in the culture medium with 1 mM 2,5-xylydine. This enzyme activity is less than half of the laccase activity obtained from SBM with 1 mM 2,5-xylydine in the present study. However, the reduced stimulation observed at a concentration above 1 mM may be due to the toxic effects of this aromatic compound on the fungal pellets. Laccase production may vary depending on the strain, cultivation condition, inducer, and the concentration of inducer used.

There are limited studies on the stimulation of laccase production by the combination of Cu and 2,5-xylydine. Tavares et al. (2005) determined that the combination

of 0.075 mM copper sulfate and 0.030 mM 2,5-xylydine enhanced the activity of *T. versicolor* laccase from 190 to 360 U/L. Cordi et al. (2007) also tested the joint effect of 0.005–0.1 mM copper sulfate with 1 mM of 2,5-xylydine on laccase production in *T. versicolor* CCT 4521, and the highest laccase activity obtained was 40774 U/L at day 12. Osmá et al. (2011) reported that the laccase activity of *T. pubescens* increased from 7751 to 19085 U/mL through the addition of Cu (0.5 mM) + xylydine (1 mM) to sunflower seed shell medium during solid-state fermentation. Revankar and Lele (2006) reported that the use of 1 mM copper sulfate and 0.8 mM 2,5-xylydine each increased laccase production considerably. Although the combination of these two inducers stimulated more laccase production than the Cu alone, the laccase activity obtained from this combination was lower than the activity detected in the medium with only 0.8 mM 2,5-xylydine. Collins and Dobson (1997) showed that when Cu, 2,5-xylydine, or both of them were supplemented to the cultures of *T. versicolor*, laccase transcription was immediately activated and laccase mRNA rapidly accumulated. However, the combination of Cu and 2,5-xylydine activated the laccase transcription faster than either separately (Collins and Dobson, 1997). The effect of the combinations of various concentrations (0.05–1.5 mM) of 2,5-xylydine with 0.5 mM Cu on laccase production was also investigated in our study. The highest laccase activities were obtained on day 7 of incubation in SBM with 0.5 mM Cu + 0.5 mM 2,5-xylydine and 0.5 mM Cu + 1 mM 2,5-xylydine as 33.61 and 31.27 U/mL, respectively.

PAGE of the *T. versicolor* ATCC 200801 culture filtrate obtained from SBM + 0.5 mM Cu + 0.05 mM ABTS was determined by SDS-PAGE and native PAGE. Coomassie Brilliant Blue staining showed an extensive laccase band in SDS-PAGE with a molecular mass of about 65 kDa (Figure 1A). An extensive laccase activity band was also observed in native PAGE (Figure 1B). The molecular weights of different laccases may vary depending on the type and even strain of fungi producing them. The majority of fungal laccases have a molecular mass between 60 and 80 kDa (Giardina et al., 2010). The combination of the inducers may affect the synthesis of the laccase isozymes. Here, PAGE analysis of the culture filtrates obtained from other media with the combination of inducers was not investigated.

The results show that it is possible to stimulate laccase production in *T. versicolor* ATCC 200801 under repeated-batch conditions through the addition of suitable concentrations of inducers. The efficiency order of these inducers is 0.5 mM Cu > 0.05 mM ABTS > 1 mM 2,5-xylydine > 1 mM guaiacol > 0.1 mM syringaldazine (Table 2). The combination of Cu with other inducers stimulated great amounts of laccase production in this

strain under repeated-batch conditions. The efficiency order of their combinations with 0.5 mM Cu is 0.5 mM 2,5-xylidine > 0.05 mM ABTS > 0.1 mM syringaldazine > 1 mM guaiacol (Table 2). Therefore, very high amounts of laccase can be obtained by selecting suitable inducer combinations and concentrations. To the best of our knowledge, this is the first report on the effect of inducer combinations on laccase production in *T. versicolor* ATCC 200801 strain under repeated-batch conditions. The repeated-batch process is easy, cheap, and a suitable

method for high amounts of laccase and repeated laccase production. Stimulation of laccase production is possible by selecting an appropriate inducer concentration, strain, production method, and, in particular, an effective inducer combination.

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