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Research Article/Araştırma Makalesi

Laccase Production of Newly Isolated *Trametes versicolor* under Batch, Repeated-Batch, and Solid-State Fermentation Processes

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Abstract: In this study, the laccase production ability of the newly isolated *Trametes versicolor* strain was investigated in three different fermentation processes. In all three fermentation processes, the fungus was able to produce the laccase enzyme. During the solid-state fermentation process 13.21 U/mL laccase activity was detected on the 20th day in the 10 mM copper-containing medium, while this value reached to 27.30 U/mL in the medium containing 0.5 mM ABTS+10 mM copper. During the liquid batch fermentation process, laccase activity was significantly induced in the medium containing 1 mM copper and the laccase activities reached 2.25, 19.83 and 24.57 U/mL compared to the medium without copper on the 3rd, 6th, and 9th days, respectively. ABTS and xylidine induced the laccase production of this strain at a much lower level than copper. The liquid repeated-batch process also significantly induced the laccase production. While low level of enzyme activities were detected in a copper-free medium, laccase activities were induced in the copper-containing medium and the activity increased from 0.66 U/mL to 9.87 U/mL at the 6th use of the pellets. Copper was detected as an effective inducer for laccase production in all fermentation processes and activity staining after native polyacrylamide gel electrophoresis clearly showed the active laccase bands. The results revealed that this strain is a good laccase producer and the laccase production yield varies depending on the fermentation process, production time, and inducer used.

Keywords: Copper, enzyme, incubation, inducer, white rot fungus, zymogram.

Yeni İzole Edilen *Trametes versicolor*'un Kesikli, Tekrarlı-Kesikli ve Katı-Faz Fermentasyon Süreçlerinde Lakkaz Üretimi

Öz: Bu çalışmada yeni izole edilmiş *Trametes versicolor* suşunun lakkaz üretim yeteneği üç farklı fermentasyon sürecinde araştırılmıştır. Üç fermentasyon sürecinde de fungus lakkaz enzimini üretebilmiştir. Katı-faz fermentasyonu sürecinde 10 mM bakır içeren ortamda üretimin 20. gününde 13.21 U/mL lakkaz aktivitesi izlenmiştir, bu değer 0.5 mM ABTS+10 mM bakır içeren ortamda ise 27.30 U/mL'ye ulaşmıştır. Sıvı kesikli fermentasyon sürecinde ise 1 mM bakır içeren ortamda bakır içermeyen ortama göre lakkaz aktivitesi önemli oranda indüklenmiş ve lakkaz aktiviteleri 3, 6 ve 9. günlerde sırasıyla 2.25, 19.83 ve 24.57 U/mL'ye ulaşmıştır. ABTS ve ksilidin bu suşun lakkaz üretimini bakıra göre çok daha düşük düzeyde indüklemiştir. Sıvı tekrarlı-kesikli süreç de lakkaz üretimini önemli oranda indüklemiştir. Bakır içermeyen ortamda düşük düzeyde enzim aktiviteleri saptanırken, bakır içeren ortamda lakkaz aktiviteleri indüklenmiş ve peletlerin 6. kullanımında aktivite 0.66 U/mL'den 9.87 U/mL'ye ulaşmıştır. Tüm fermentasyon süreçlerinde, bakırın lakkaz üretimi için önemli bir indükleyici olduğu gözlenmiştir ve doğal poliakrilamid jel elektroforezi sonrası yapılan aktivite boyamaları aktif lakkaz bantlarını net olarak göstermiştir. Sonuçlar, bu suşun iyi bir lakkaz üreticisi olduğunu ve fermentasyon sürecine, üretim zamanına ve kullanılan indükleyiciye bağlı olarak lakkaz üretim veriminin değiştiğini ortaya koymuştur.

Anahtar kelimeler: Bakır, enzim, inkübasyon, indükleyici, beyaz çürükçül fungus, zimogram.

1. Introduction

Laccase enzyme was first discovered by Yoshida in 1883 in the secretions of the Japanese lacquer tree called *Rhus vernicifera* and was described as a metal-containing oxidase (Thurston, 1994; Mayer & Staples, 2002). White rot fungi, bacteria, plants, and insects can produce laccase enzyme. This enzyme, which has low substrate specificity, is commonly found in white rot fungi (Eggert et al., 1996; Upadhyay et al,. 2016). Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases with phenol oxidase activity and due to their low substrate specificity, they can oxidize many different substrates (Yeşilada et al., 2014; Agrawal et al., 2018; Moreno et al., 2020). They can be used in different areas and processes (Yeşilada et al., 2014, Forootanfar & Faramarzi, 2015; Rodríguez-Delgado et al., 2016; Dhull et al., 2020;).

Laccase can be produced by different fermentation processes such as solid-state fermentation (SSF), liquid batch fermentation (LBF), and liquid repeated-batch fermentation (LRBF). SSF is a medium in which there is no free liquid in the medium. That is, organisms can grow and carry out their metabolic reactions using moistened solid substrates (Pandey et al., 2000; Krishna, 2005). Lignocellulose substrates are abundant in nature and some of them are in the form of waste materials. White rot fungi can use lignocellulose raw materials as substrate. These fungi can degrade cellulose, hemicellulose, and lignin in lignocellulosic substrates thanks to their enzymes such as laccase, ligninase, cellulase, pectinase, and xylanase (Pandey et al., 2000; Papinutti et al., 2007; Boran & Yeşilada, 2011). SSF is a preferred application in the production of important products as it resembles the

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natural habitats of the filamentous fungi. Bacteria, yeasts, and filamentous fungi can be grown on solid substrates. However, the most suitable microorganisms for SSF processes are fungi that grow well in humid environments (Manan & Webb, 2017). Many different substrates can be used in SSF. These substrates include wheat bran, straw, various fruit peels, plant leaves, and sawdust (Rosales et al 2007; Elisashvili et al 2008; Levin et al 2008; Sharma & Arora, 2010; Boran & Yeşilada, 2011). The batch process is a production process in which no fresh substrate is added to or removed from the culture until the end of production, after the microorganism has been inoculated into a growth medium. In the repeated-batch process after inoculation and incubation, a certain amount of the culture liquid is removed and the same amount of fresh medium is added. Microorganisms remain in the environment throughout the process and the process is repeated as many times as desired. In this process, microorganisms can be used repeatedly for a long time (Birhanli & Yesilada, 2010).

In this study, the laccase production ability of the newly isolated *Trametes versicolor* strain was investigated under different fermentation processes and the effect of various inducers on laccase production ability of this strain was tested.

2. Material and Methods

2.1. White Rot Fungus Used

Trametes versicolor, a white rot fungus belonging to the Basidiomycetes class, was used in the study. This fungus was collected from Hatay by Dr. Özfer Yeşilada and identified after being isolated as a pure culture. The fungus used in the study is maintained as a pure culture in the Biotechnology Laboratory of the Department of Biology at İnönü University.

2.2. Macroscopic Determination of Laccase Enzyme Production of *Trametes versicolor*

Laccase production ability of this fungus was detected on SDA plates containing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS). Firstly, the fungus inoculated on this plate and then the culture was statically incubated at 30°C. The color change due to ABTS oxidation shows the laccase production ability of this fungus (Boran & Yeşilada, 2022).

2.3. Preparation of Stock Inoculum Culture

Firstly, the fungus inoculated on slant SDA medium was incubated at 30 °C for 5 days. Then, distilled water was added onto this culture and mycelial suspension was obtained. After that, 4 mL of this suspension was inoculated into 100 mL Sabouraud dextrose broth (SDB)/250 mL flask and it was incubated at 30 °C and 150 rpm for 7 days. After incubation, the obtained liquid culture was homogenized at low speed with a homogenizer under sterile conditions and 4 mL of this homogenized culture was inoculated into 100 mL SDB/250 mL flasks. This culture was incubated at 30 °C and 150 rpm for 7 days. After production, this liquid culture was gently homogenized at low speed and used as stock inoculum culture (Yeşilada et al 2014).

2.4. Preparation of Fungal Pellets for Repeated-Batch Fermentation

In order to obtain the fungal pellets, 600 mL SDB/1000 mL flask was prepared first and it was autoclaved at 121 °C for 20 minutes. 7 mL of the homogenized stock inoculum culture prepared as stated in Section 2.3, was inoculated into this broth. The culture was incubated for 7 days at 30 °C and 150 rpm for the formation of fungal pellets. Then, the pellets were filtered under sterile conditions, washed with sterile distilled water, and the fungal pellets obtained were used for repeated-batch studies.

2.5. The Fermentation Methods Used

2.5.1. Batch fermentation

Batch fermentation (BF) studies were carried out in 50 mL SDB/250 mL flasks. The effect of various inducers on laccase production was also tested in batch studies. For this purpose, the media containing 1 mM copper, 1 mM xylidine, 0.05 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1 mM xylidine+1 mM copper, and 0.05 mM ABTS+1 mM copper (final concentrations) and the media without an inducer were prepared. These media were inoculated with 1 mL of the homogenized stock inoculum culture and they were incubated at 30°C and 150 rpm for 9 days.

2.5.2. Repeated-batch fermentation

Fungal pellets obtained as stated in Section 2.4 were used in the repeated-batch fermentation (RBF). 30 grams of pellets were transferred to 50 mL SDB/250 mL flasks with and without inducer under sterile conditions. After the cultures were incubated for 24 hours at 30 °C and 150 rpm, the culture liquids were filtered and fresh sterile media were added to these flasks containing pellets and incubated for 24 hours in the same way (Birhanlı & Yeşilada, 2010). These studies were carried out with medium without inducer and also in media containing 1 mM copper, 1 mM xylidine, 0.05 mM ABTS, 1 mM xylidine+1 mM copper, and 0.05 mM ABTS+1 mM copper separately.

2.5.3. Solid-state fermentation

For the preparation of solid-state medium, 3.5 g wheat bran+1.5 g soy flour were transferred into 250 mL flasks and these solid media were moistened with 15 mL of moistening liquid (sterile distilled water or sterile distilled water containing the appropriate inducer). Prepared solid media were autoclaved at 121°C for 20 minutes. Then, 4 mL of homogenized stock culture prepared as specified in Section 2.3 was inoculated into these solid media and they were statically incubated at 30°C. After incubation, 40 mL of sterile distilled water was added to each solid-state cultures and the cultures were mixed with sterile sticks. Then, the cultures were shaken at 30°C and 150 rpm for 2 hours. After shaking, the cultures were filtered and the obtained filtrates were centrifuged twice for 10 minutes at 7000 rpm. Laccase enzyme activity was determined in the supernatants obtained after centrifugation (Boran & Yeşilada, 2011).

2.6. Determination of Laccase Activity

For the determination of laccase (EC 1.10.3.2) activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

was used as a substrate and laccase activity was determined depending on ABTS oxidation by laccase enzyme. The measurement of laccase activity was carried out at 40 °C and the reaction mixture was prepared with sodium acetate buffer (100 mM, pH 4.0), ABTS (5 mM), and the appropriate amount of supernatant. As a result of the oxidation of ABTS, the absorbance change occurring in 1 minute at 420 nm was determined and the amount of enzyme that converts 1 µmol of substrate to product in 1 minute at 40°C was expressed as 1 unit (Yeşilada et al., 2014). All laccase activity values are given as the mean of at least three studies.

2.7. Native Gel Electrophoresis

Native gel electrophoresis was applied to show the presence of laccase enzyme. TGX gels were used. The crude enzymes were loaded into the wells and were run at 40 mA. After the electrophoresis, the gel was incubated in pH 4.0 acetate buffer containing ABTS and activity staining was performed (Yeşilada et al., 2014).

3. Results and Discussion

White rot fungus *Trametes versicolor* is a biotechnologically important fungus. It is also a medicinal fungus (Kılıç & Yeşilada, 2013; Winder et al., 2021). *T. versicolor* was also the effective producer of the laccase enzyme. Since the laccase enzyme can be used in many applications, it is important to produce this enzyme in high amounts. The laccase production potential of white rot fungi may vary depending on the species used and even on the strain. Therefore, in this study, the laccase production ability of *Trametes versicolor*, a white rot fungus isolated from Hatay province, was investigated.

3.1. Detection of Laccase on Sabouraud Dextrose Agar Media containing ABTS

T. versicolor was cultivated on SDA media with and without ABTS. If the fungus is able to oxidize ABTS, the color of the medium changes to green-purple. As shown in Figure 1 the fungus was able to oxidize ABTS and a purple color was formed. This is an indication that this fungus is a producer of laccase.

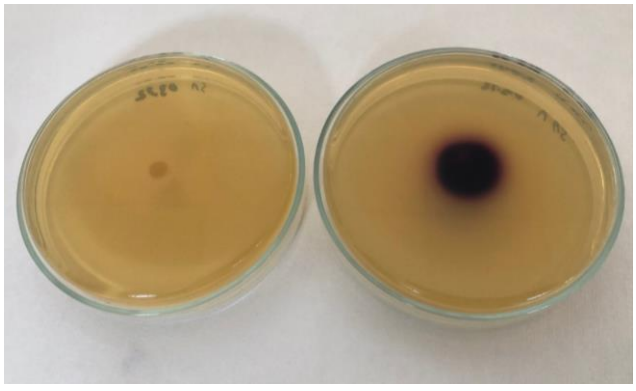

Figure 1. Color change in SDA media due to ABTS oxidation. a) ABTS-free medium, b) ABTS-containing medium.

3.2. Laccase Production of *T. versicolor* Under Different Fermentation Processes

The laccase production potential of white rot fungi could vary depending on the fermentation processes (solid-state fermentation, liquid batch fermentation, and liquid repeated-batch fermentation).

3.2.1. Laccase production during solid-state fermentation process

During the SSF, wheat bran+soy flour (3.5 g+1.5 g) was used as solid substrate. After this solid substrate was prepared, it was moistened with distilled water and sterilized in an autoclave (Boran & Yeşilada, 2011). After sterilization, the fungus was inoculated and they were statically incubated at 30°C for 20 days. Firstly, laccase production ability of this fungus was monitored by adding ABTS to the solid culture. After ABTS addition, the formation of green color due to the oxidation of ABTS showed the laccase production of this fungus under SSF (Fig. 2).

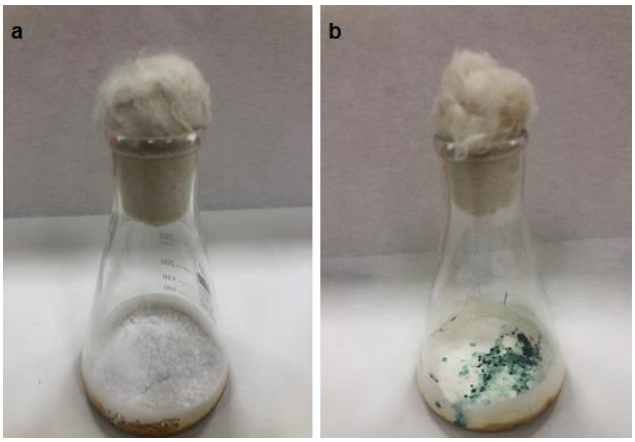

Figure 2. The macroscopic images of *T. versicolor* incubated on (a) solid-state medium and (b) solid-state medium treated with ABTS after incubation.

After the macroscopic observation, the effect of incubation time (5, 10, 15 and 20 days) on laccase production of this fungus was tested. As seen in Figure 3, while the laccase activity was determined as 0.17 U/mL on the 5th day, the laccase activity increased with time and reached 10.70 U/mL on the 20th day. The laccase production potential of white rot fungi under SSF was reported in other studies (Boran and Yeşilada, 2011).

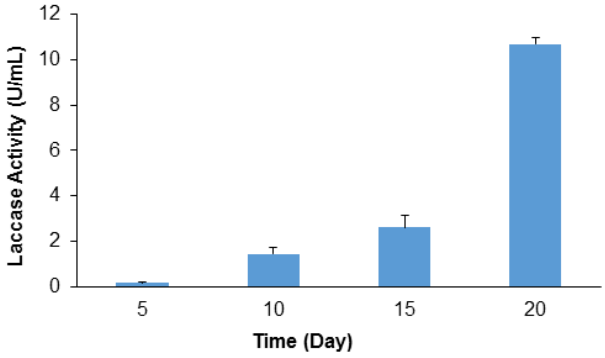

Figure 3. Time-dependent laccase production during SSF.

3.2.2. Effect of inducers on laccase production during solid-state fermentation

It has been reported in various studies that inducers affect the laccase production of white rot fungi (Collins & Dobson, 1997; Boran & Yeşilada, 2011; Birhanlı & Yeşilada, 2017). Therefore, the effect of inducers such as copper, xylidine, and ABTS on laccase production during solid-

state fermentation was tested. In addition, the combined effect of copper+xylidine and copper+ABTS was also investigated. It was reported that copper is a good inducer for laccase production (Birhanlı & Yeşilada, 2006). The addition of copper to the medium increased the laccase activity. While the laccase activity detected on the 20th day was 13.21 U/mL in the copper-containing medium, it was 10.70 U/mL in the copper-free medium. Laccase activities detected on solid media moistened with distilled water containing 10 mM copper were 0.74, 3.87, 6.79 and 13.21 U/mL on the 5th, 10th, 15th, and 20th days, respectively (Fig. 4).

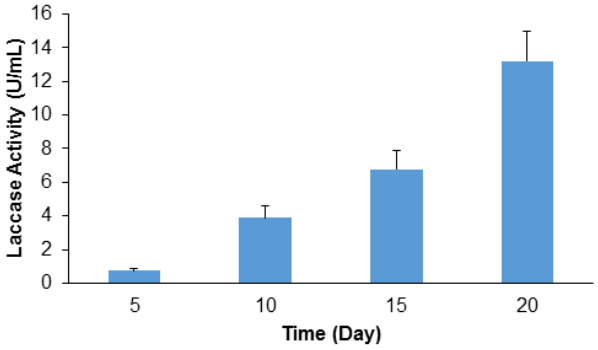

Figure 4. Time-dependent laccase production in 10mM copper-containing medium during SSF.

The effect of ABTS and xylidine on laccase production of this fungus was also tested. In addition, the combined effect of ABTS+copper and xylidin+copper was also tested in order to test their possible synergistic effect with copper. For this purpose, the fungus was incubated the solid media containing these inducers for 20 days and laccase activities were measured. While 5.60 U/mL laccase activity was obtained in 0.5 mM ABTS medium, 27.30 U/mL laccase activity was detected in 0.5 mM ABTS+10 mM copper medium (Fig. 5). Furthermore, while 5.53 U/mL laccase activity was detected on the 20th day in the medium containing 10 mM xylidine, 22.59 U/mL enzyme activity was determined in the medium containing 10 mM xylidine+10 mM copper (Fig. 6).

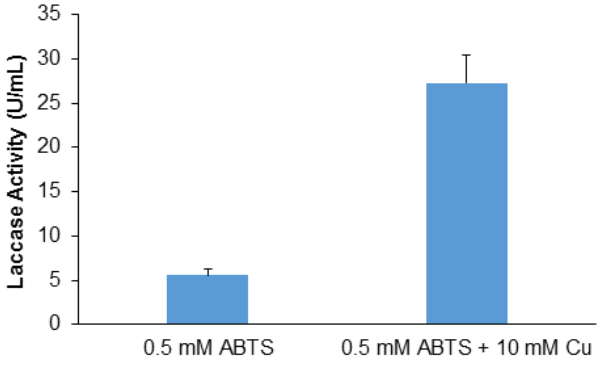

Figure 5. Time-dependent laccase production in 0.5 mM ABTS and 0.5 mM ABTS+10mM copper-containing media during SSF.

3.2.3. Laccase production under liquid phase fermentation

Laccase production potential of *T. versicolor* was investigated during the liquid phase fermentation processes (batch and repeated-batch processes).

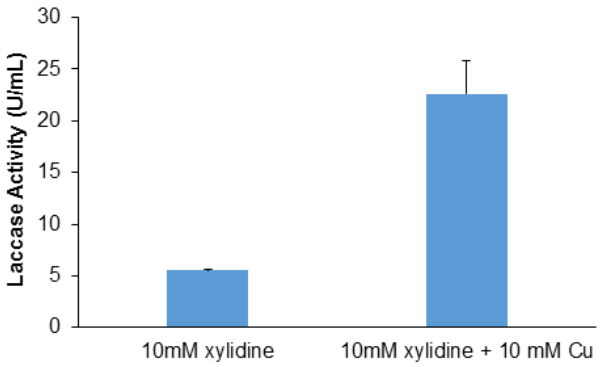

Figure 6. Time-dependent laccase production in 10mM xylidine and 10 mM xylidine+10mM copper-containing media during SSF.

3.2.3.1. Laccase production during batch fermentation

Time-dependent laccase production monitored for 9 days. As seen in Figure 7, laccase activities were significantly lower in the SDB medium without any inducer. The fungus produced 0.033, 0.026, and 0.013 U/mL laccase enzymes after incubation for 3, 6, and 9 days in copper-free SDB media, respectively. On the other hand, laccase production was significantly induced in the media containing 1mM copper and laccase activities reached 2.25, 19.83 and 24.57 U/mL on the same days (Fig. 8). The laccase activity increased 68 times on the 3rd day, 762 times on the 6th day and 1890 times on the 9th day with the addition of copper compared to the cultures growth in copper-free media. This result shows that copper plays an important role as an inducer of laccases during batch fermentation (Lorenzo et al., 2006; Cordi et al., 2007).

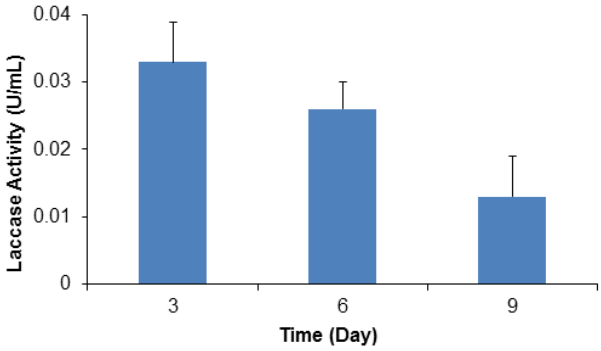

Figure 7. Time-dependent laccase production in SDB medium without inducer during BF.

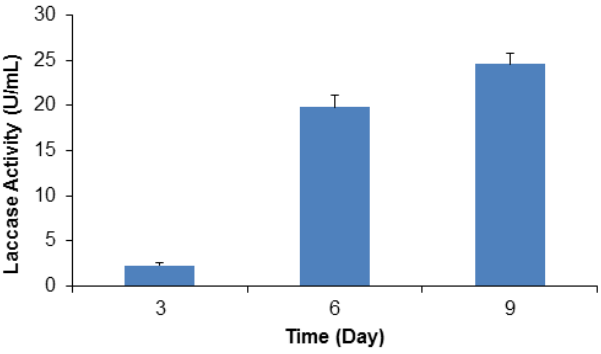

Figure 8. Time-dependent laccase production in 1 mM copper-containing SDB medium during BF.

In addition to copper, the effect of ABTS and xylidine on laccase production after 6 days of incubation was tested. In SDB medium containing 0.05 mM ABTS, 0.17 U/mL laccase activity was detected. On the other hand, this value was 0.16 U/mL in SDB medium containing 1 mM xylidine (Fig. 9). These results showed that these inducers slightly induce the laccase production activity of this fungus under BF condition. Copper was detected as the best inducer for this fermentation process (Fig. 7-Fig. 9).

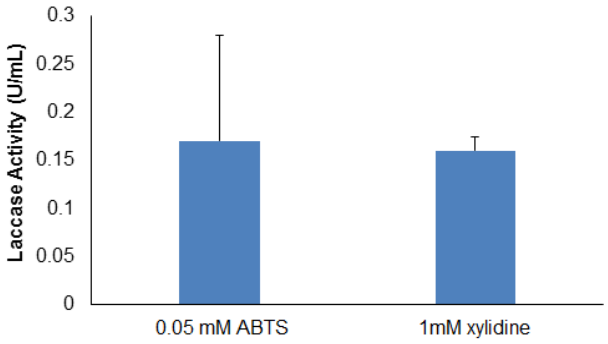

Figure 9. Time-dependent laccase production in SDB media containing 0.05 mM ABTS and 1 mM xylidine during BF.

Laccase production ability of this fungus was tested in media containing 0.05mM ABTS+1mM copper and also media containing 1mM xylidin+1mM copper. While 10.50 U/mL laccase activity was detected on the 6th day in the medium containing 0.05 mM ABTS+1 mM copper, 10.69 U/mL laccase activity was obtained in the medium containing 1 mM xylidin+1 mM copper.

3.2.3.2. Laccase production during repeated-batch fermentation

During the RBF, firstly, the effect of copper as an inducer was tested. As can be seen from Figure 10, low-level enzyme activities were detected in the copper-free medium. However, laccase production was significantly induced in the copper-containing medium. Laccase production increased from the 1st use of the pellets until the 6th use, in a copper-containing medium. Laccase production ability of the pellets decreased after the 6th use. However, the laccase activity was high even at the 8th use. This shows that these self-immobilized pellets can be used for repeatedly and long-term production of laccase could be achieved. This is consistent with studies reporting the positive effect of copper on laccase production during repeated-batch process (Birhanlı & Yeşilada, 2017).

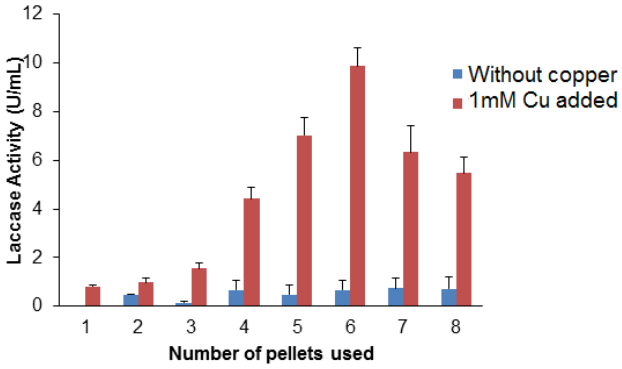

Figure 10. Time-dependent laccase production in 1 mM copper-containing and copper-free SDB media during RBF.

The effect of ABTS and xylidine, as inducers on laccase production, during the repeated-batch process was also investigated. 0.05 mM ABTS showed no positive effect on laccase production. Although xylidine has a positive effect on laccase production, this effect is negligible compared to copper (Fig. 11).

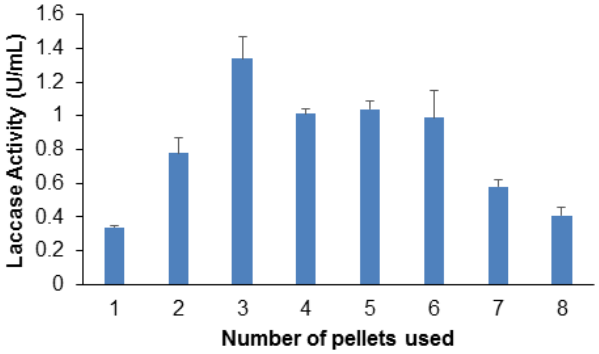

Figure 11. Time-dependent laccase production in 1 mM xylidine-containing medium during RBF.

The possible synergistic effect of 0.05 mM ABTS+1 mM copper and 1 mM xylidine+1 mM copper was also tested. No significant increase was observed in the medium containing 0.05 mM ABTS+1 mM copper compared to the medium containing only copper (Fig. 12). As can be seen in Figure 13, laccase activity in the medium containing 1 mM xylidine+1 mM copper increased during the first 5 uses and after that, the activity decreased. In previous studies, a synergistic effect of inducers such as ABTS+copper or xylidine+copper on laccase production on fungi was reported (Birhanlı & Yeşilada, 2017).

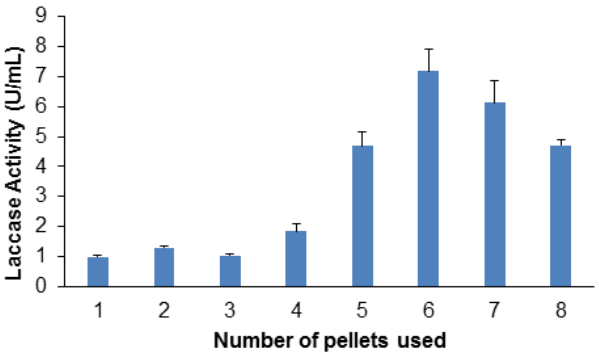

Figure 12. Time-dependent laccase production in SDB medium containing 0.05 mM ABTS+1 mM copper during RBF.

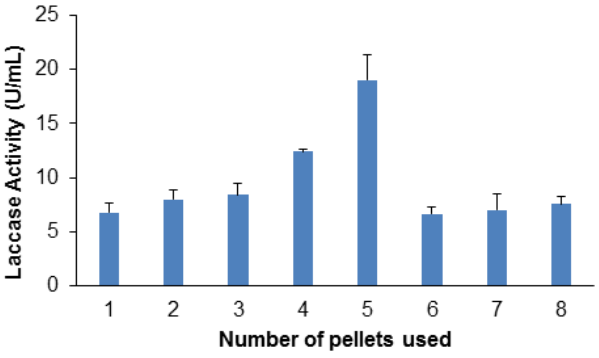

Figure 13. Time-dependent laccase production in SDB medium containing 1 mM xylidine+1 mM copper during RBF.

3.3. Zymogram Studies

Zymogram applications of enzyme sources obtained from SSF, BF, and RBF processes were made. After the electrophoresis, the activity staining was done using ABTS and the gel was photographed. As can be seen from Figure 14, active laccase bands were observed in all fermentation conditions.

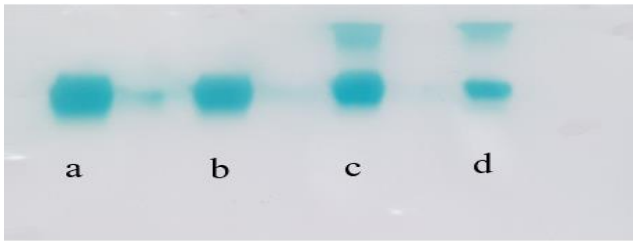

Figure 14. Detection of laccase on native PAGE gels (a) Enzyme source from copper-free 20th day culture of SSF (b) Enzyme source from 10 mM copper-containing 20th day solid-state culture (c) Enzyme source from 1 mM copper-containing 9th day batch culture (d) Enzyme source from 1 mM copper-containing repeated-batch culture (at the 6th use of pellets).

4. Conclusions

The fermentation process, time, and inducer selection significantly affected the laccase production potential of this *T. versicolor* strain. In all three fermentation processes, this fungus was able to produce the laccase enzyme. Copper was detected as an effective inducer for laccase production in all fermentation processes. The highest laccase activity was determined in SSF medium with 0.5 mM ABTS + 10 mM Cu. The high level of biotechnologically important laccase enzyme production with this strain shows that this strain could be used as an effective laccase producer by selecting the proper inducer and fermentation mode.

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Ethics committee approval: Ethics committee approval is not required for this study.

Conflict of interest: The author declared that there is no conflict of interest.

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