Araştırma Makalesi
Adıyaman Üniversitesi Mühendislik Bilimleri Dergisi
17 (2022) 458-470

LACCASE PRODUCTION BY NEWLY ISOLATED GANODERMA LUCIDUM WITH SOLID STATE FERMENTATION CONDITIONS AND ITS USING FOR DYE DECOLORIZATION

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Geliş Tarihi/Received Date: 22.04.2022 Kabul Tarihi/Accepted Date: 20.05.2022 DOI: 10.54365/adyumbd.1107682

ABSTRACT

Laccase production capacity of newly isolated fungus *Ganoderma lucidum* was studied during solid state fermentation on solid substrates. Because wheat bran was detected as the most effective solid substrate, effect of various culture parameters and some inducers on laccase production in wheat bran media was investigated. Wheat bran with moisture content of 75%, pH 5.0 and 30 °C was the most effective medium and 10 mM copper addition supported the highest amount of laccase. In glass tray fermenter 2973±220 U/L laccase activity was obtained. Crude laccase gave the highest activity at 70 °C. It was stable at 60°C for 6 hours and retained 37% activity at 70 °C for 2 hours. It decolorized 61% of Remazol Brilliant Blue R within 20 min. Results showed that this strain could produce high amount of laccase and suitable inducer such as copper could induce its laccase production. The crude laccase obtained could be used for textile dye decolorization applications.

Keywords: Decolorization; Ganoderma lucidum; Laccase; Solid state fermentation; Wheat bran

YENİ İZOLE EDİLMİŞ GANODERMA LUCIDUM İLE KATI HAL FERMANTASYONU KOŞULLARINDA LAKKAZ ÜRETİMİ VE BOYA RENK GİDERİMİNDE KULLANILMASI

ÖZET

Yeni izole edilmiş fungus *Ganoderma lucidum*' un lakkaz üretim kapasitesi, katı substratlar üzerinde katı ortam fermantasyonu sırasında incelenmiştir. Buğday kepeğinin en etkili katı substrat olduğu tespit edildiğinden, buğday kepeği ortamında çeşitli kültür parametrelerinin ve bazı indükleyicilerin lakkaz üretimine etkisi araştırılmıştır. pH 5.0, 30 °C ve %75 nem içeriği ile buğday kepeği en etkili ortamdı ve 10 mM bakır ilavesi en yüksek lakkaz miktarını destekledi. Cam tava fermentörde 2973±220 U/L lakkaz aktivitesi elde edildi. Ham lakkaz 70 °C'de en yüksek aktiviteyi vermiştir. 60°C'de 6 saat stabildi ve 70°C'de 2 saat boyunca %37 aktiviteyi korudu. Ham lakkaz, 20 dakika içinde Remazol Parlak Mavi R' nin %61 rengini giderdi. Sonuçlar, bu suşun yüksek miktarda lakkaz üretebileceğini ve bakır gibi uygun indükleyicilerin lakkaz üretimini indükleyebileceğini gösterdi. Elde edilen ham lakkaz, tekstil boyası renk giderme uygulamaları için kullanılabilir.

Anahtar Kelimeler: Renk giderimi; Ganoderma lucidum; Lakkaz; Katı ortam fermentasyonu; Buğday kepeği

1. Introduction

Solid state fermentation (SSF) is a method processing in the absence or near absence of free water with solid substrate [1]. The use of low-cost and ecofriendly solid substrates or wastes is important for the cost of the application and also for the reduction of environmental pollution. Lignocellulosic

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solid substrates such as wheat cob or straw, sugar cane waste, coffee waste, grape waste and wheat bran are the major substrates for this type of fermentation [2].

SSF could be used to produce many industrial enzymes [3] and this process could be a promising technology for laccase production in high amounts and low-cost. Species and even the strain, culture condition, substrate and inducers are important for laccase production with fungi during SSF.

Laccase oxidizing various compounds including xenobiotics are produced by white rot fungi. Various studies reported the laccase production by white rot fungi [4, 5]. It is important to produce this enzyme at a high level with an appropriate method and inexpensive way [6]. Lignocellulosic raw materials/wastes containing various substrates and inducers for laccase induction may be a suitable alternative as a solid substrate for laccase production [7, 8].

It is hard to decolorize the textile dyes by conventional biological methods. White rot fungi are the important organisms with their high dye decolorization capacity [9]. They produce laccase enzyme which is the main biological system for their dye decolorization activity [10]. *Ganoderma lucidum* is medicinal white rot fungus and produces this biotechnologically important enzyme as a main ligninolytic enzyme [11, 12].

The aim of this study was to test the laccase production ability of *Ganoderma lucidum* during SSF by using different supports/solid substrates and also to determine the effect of moistening agent, moisture content, culture conditions and inducers on laccase production. Moreover, laccase production capability of this fungus in a model of tray type fermenter was also tested under optimum condition. The activity and stability of the crude laccase was determined and its dye decolorization potential was also investigated using Remazol Brilliant Blue R (RB19).

2. Materials and methods

2.1. Fungal strain

Newly isolated *Ganoderma lucidum* was used as the main fungal strain in the study. This strain was cultivated on Sabouraud Dextrose Agar (SDA) plates at 4 °C and sub-cultivated monthly.

2.2. Solid substrates

Various agro-industrial wastes and residues like wheat bran, waste leaves (*Platanus orientalis*), poplar sawdust, walnut shell, pine cone, wheat straw and corn cob were used. They were dried at 50 °C before the experiments.

2.3. Moistening agent

Sterile distilled water was used directly or it was prepared as distilled water containing yeast extract (1, 5 and 20 g/L) or distilled water containing $CuSO_4.5H_2O$ (1, 5 and 10 mM) for testing the possible role of yeast extract and copper.

2.4. Preparation of inoculum, solid media and solid state fermentation

The SSF cultivations were performed in flasks containing 5 g wheat bran/15 mL distilled water, 10 g walnut shell/7mL distilled water, 5 g pine cone/10mL distilled water, 5 g waste leaves/15mL distilled water, 4 g poplar sawdust/15mL distilled water, 3 g corn cob/15mL distilled water and 3 g wheat straw/15mL distilled water. To test the effect of soy flour or waste leaves on laccase production, wheat bran+soy flour (WB+SF) media or wheat bran+waste leaves media were prepared in different

ratios (1:1, 2:1, 4:1, 9:1) and moistened with the moistening agent. All these media were autoclaved at 121°C for 45 min.

The solid media were inoculated with the homogenized mycelial pellets. For preparing these homogenized mycelial pellets, *G. lucidum* incubated in a 100 mL volume of Sabouraud dextrose broth (SDB) for 5 days was homogenized and the prepared solid media, as stated above, were inoculated with 2 mL of these homogenized mycelial pellets. The cultures were incubated statically at 30 °C for 5 days unless otherwise indicated. After incubation period, distilled water in a volume of 40 was added into solid cultures. Then these cultures were agitated at 200 rpm and 30 °C for 1 hour. After that, they were filtered and centrifuged. The obtained supernatants were used for detection the laccase activity. The effect of temperature (20 °C-40 °C), pH (3.0-7.0) and moisture content (50–85%) on laccase production was also tested.

Tray type fermenter was also tested for laccase production. Glass container in the dimension of 15 cm x 27 cm was used as a tray type fermenter. The wheat bran+soy flour (w/w, 1:1) media were used and these media were moistened with distilled water or distilled water containing 10 mM copper. These prepared media were autoclaved at 121 °C for 45 min. Then, the media were inoculated with 20 mL of inoculum prepared as stated above and they were incubated statically at 30 °C for 5 days. After the incubation, 400 mL of distilled water was added into the solid cultures and they were agitated at 30 °C and 200 rpm for 1 hour. After that, these cultures were filtered, centrifuged and supernatants were utilized for detection the laccase activity.

2.5. Detection of laccase production by agar plate method

Laccase production ability of this strain was assayed on Sabouraud Dextrose Agar medium containing 0.5 mM 2,2'-azinodi-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS). The mycelia in 10 mm diameter was inoculated on agar plates containing ABTS and then they were incubated at 30 °C. The laccase production ability of this fungus was determined according to the color production based on the ABTS oxidation.

2.6. Laccase activity assay

Laccase activity was measured by the oxidation of ABTS at 420 nm. The reaction was monitored using 100 µL of 0.5 mM ABTS in the reaction mixture including sodium acetate buffer (100 mM, pH 5.0) and appropriate amount of supernatant. The enzyme amount oxidizing 1 µmol substrate in a minute was expressed as 1 unit [5, 13].

2.7. Effect of various parameters on activity and stability of crude laccase

The optimum pH of this crude laccase was assayed in a pH ranging from 2.0 to 7.0 at 30 °C for a minute. For pH stability experiments, it was firstly incubated in the range of 3.0-9.0 pH for various times (0-24 h) and then the enzyme activity was detected.

For testing the effect of temperature on crude laccase activity, the activity was determined in different temperatures ranging from 20 °C to 85 °C for 1 minute. Its thermal stability was tested by incubating it at 4–80 °C for various times (0-24 h) and then cooled at ice bath. After cooling, the laccase activity was determined at 30 °C as stated in laccase activity assay section.

2.8. Dye decolorization by crude laccase

Dye decolorization activity of the enzyme was tested using an anthraquinone group dye, Remazol Brilliant Blue R (RBBR) (RB 19). The decolorization was determined at OD_{592}.

2.9. Effect of various parameters on dye decolorization by crude laccase

For testing the effect of pH on dye decolorization, studies were researched at varied pH values (2.5-6.0) and the decolorization activity was measured spectrophotometrically at 30 °C by using 100 mg/L dye and 100 µL of the crude enzyme. To determine the effect of temperature on dye decolorization, 100 µL of enzyme source was added into buffer (pH 3.0) containing 100 mg/L of the dye and an absorbance change was detected at different temperatures (40, 50 and 60 °C).

Different amounts of crude enzyme (50-400 µL) were used to determine the effect of enzyme amount on dye decolorization. The crude enzyme was added into the buffer (pH 3) containing 100 mg/L dye and the decolorization was determined for 20 min.

To determine the effect of dye concentration on color removal, 100 µL of enzyme was added into the buffer containing different concentrations (50-400 ppm) of RB19 (pH 3) and decolorization at 592 nm was determined at 40 °C.

Dye decolorization was expressed as % color removal against to control. All values were calculated as the mean of 3 repetitions.

2.10. Laccase determination by native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis was used in order to show the presence of laccase enzyme and for this aim, crude enzyme was loaded on gel and electrophoresis was performed at 40 mA for about 1 hour. When the electrophoresis step is completed, the gel was incubated with sodium acetate buffer (pH 4.8) containing ABTS at 40 °C for detection the laccase enzyme [4].

3. Results and discussion

3.1. Laccase production of G. lucidum on agar plate containing laccase substrate

Firstly, laccase production ability of this newly isolated strain was tested on SDA medium containing ABTS as a laccase substrate. As shown Figure 1, the purple zone occurring around the mycelium was the indication of laccase production due to the oxidation of ABTS.

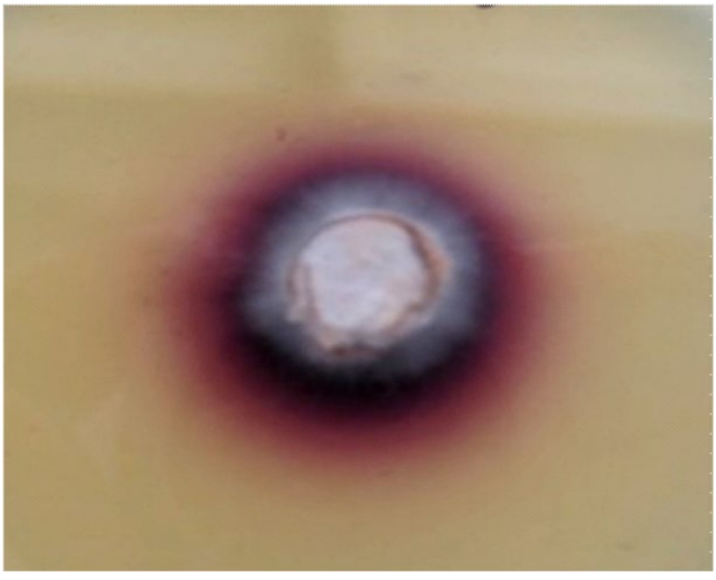

Figure 1. ABTS oxidation by *G.lucidum* on agar plates.

After understanding that this strain can produce laccase enzyme, the effect of the solid substrate, moisture content, temperature and initial pH on laccase production was investigated [5].

3.2. Effect of solid substrate on laccase production

SSF is defined as a system in which the substrate plays role as carbon and energy source. [7]. Lignocellulosic wastes/raw materials and food industry wastes are widely used as solid substrate during SSF. They serve as support materials and nutritional sources [5, 14]. Furthermore, the use of these wastes in their natural forms helps to prevent environmental pollution. Many of these wastes include lignin, cellulose and hemicelluloses, act as inducers of ligninolytic activity [15]. To determine the most effective substrate for the effective laccase production, various solid substrates such as wheat bran, waste walnut shells, waste pine cone, waste poplar sawdust, waste leaves, waste corn cobs and waste wheat straw were used. This strain grew well in wheat bran medium (Figure 2c) and wheat bran was detected as the most effective substrate for laccase production with 2814 ± 105 U/L laccase activity (Table 1). Therefore, wheat bran was chosen as the suitable substrate in the next steps of study. Figure 2 (a-d) shows photograph and scanning electron microscope (SEM) micrographs of fungus cultivated and uncultivated wheat bran media.

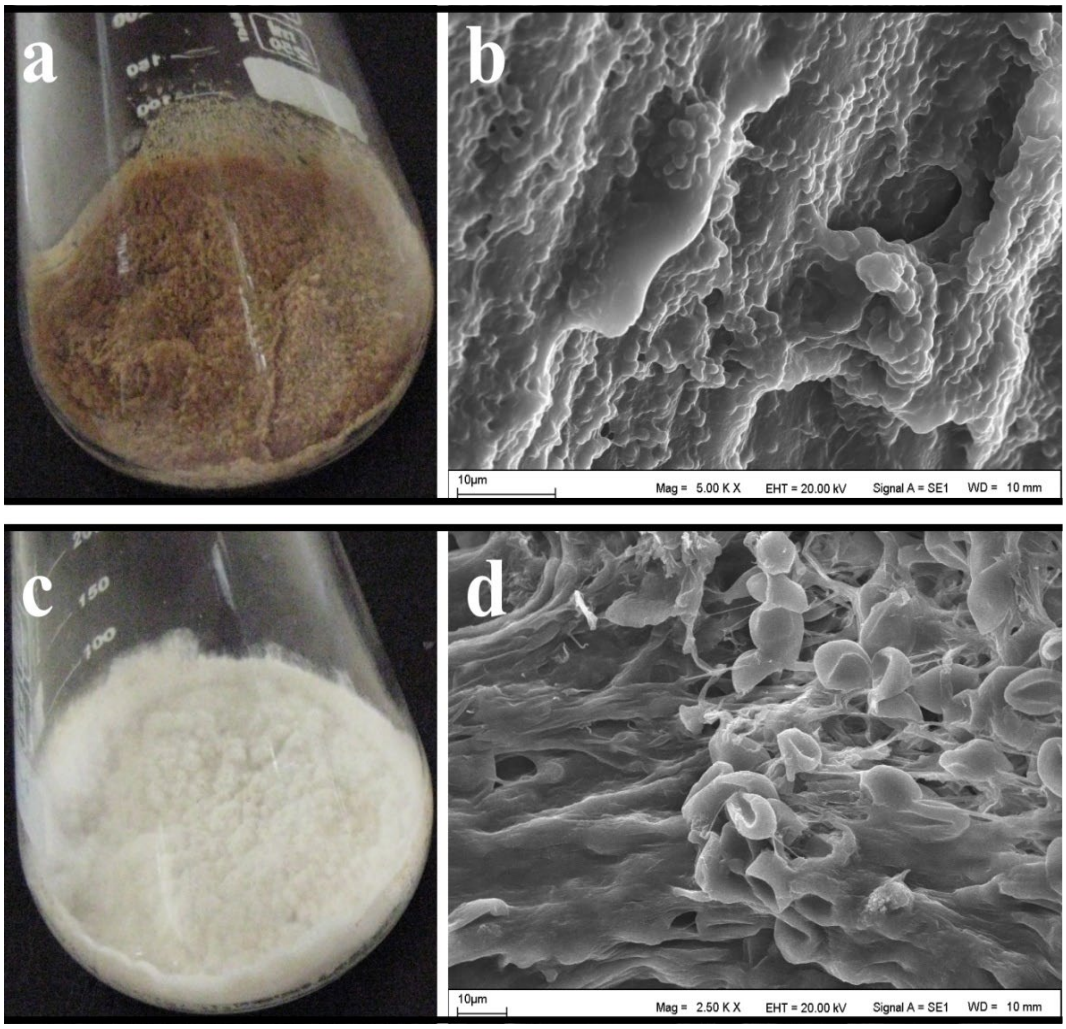

Figure 2. (a) Photograph and (b) SEM micrograph of the wheat bran moistened with distilled water, (c) photograph and (d) SEM micrograph of G. lucidum cultivated on wheat bran medium.

Table 1. Laccase activity of the solid substrate cultures of *G. lucidum* on the 5th day.

Solid Substrate	Laccase Activity (U/L)
Wheat bran	2813.7 ± 104.97
Walnut shell	104.0 ± 7.46
Pine cone	873.2 ± 114.34
Poplar sawdust	182.0 ± 26.99
Waste leave	132.0 ± 9.49
Corn cob	100.7 ± 13.35
Wheat straw	171.6 ± 10.43

Wheat bran is an important carbon source and it contains hemicellulose, starch, cellulose, protein, lignin and also phenolic compounds such as ferulic, kumaric, syringic, gentisic and caffeic acid [16, 17]. Phenolic compounds are important inducers of enzymes such as laccase [18]. It has been reported that Pleurotus pulmonarius could produce laccase during SSF with wheat bran as solid substrate [19]. The laccase activity for Ganoderma sp. was reported as 974 U/gDs in the wheat bran medium under SSF conditions [20].

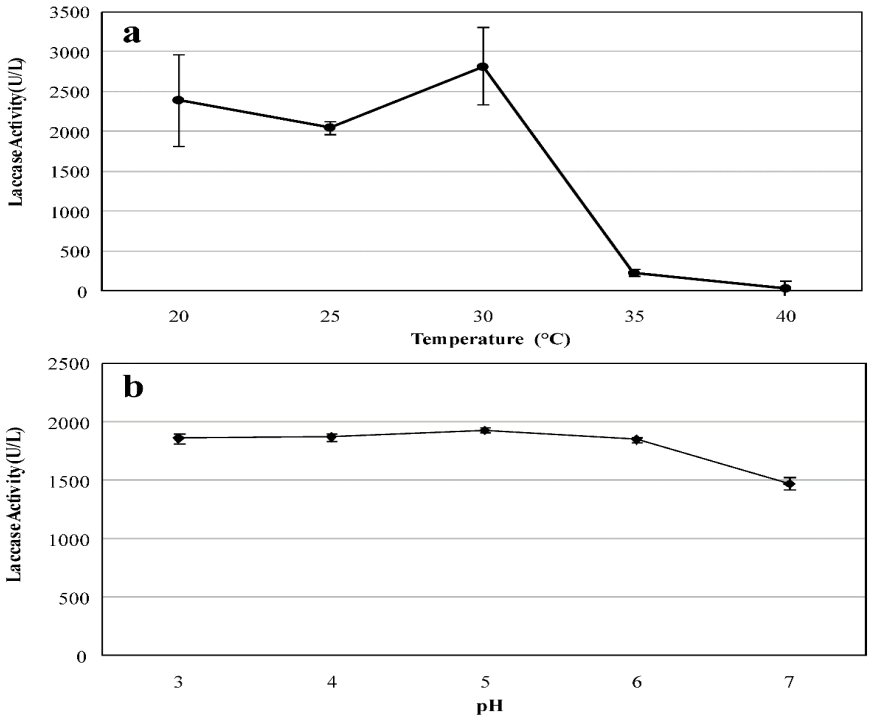

Figure 3. Effect of (a) temperature and (b) pH on laccase production of *G. lucidum* on wheat bran medium moistened with distilled water, after 5 days of incubation.

3.3. Effect of some physiological parameters on laccase production

Temperature, pH and moisture content are significant factors for fungal laccase production during SSF. Because temperature is a critical factor for enzyme production, temperature values ranging 20-40 °C were tested and the optimum temperature was determined as 30 °C (Figure 3a). pH of the medium is an another important factor for SSF. Filamentous fungi optimally grows between pH 3.8 and 6.0 [14] and each microorganism has the optimum pH range for high amount of enzyme production. In this study, pH ranging of 3.0-7.0 was tested and high enzyme activities were obtained at all pH values tested (Figure 3b).

SSF involves the microbial growth process on solid substrates without free water. Optimization of initial moisture level in SSF is important for substrate utilization and laccase production [21]. In this study, the highest laccase activity was detected on wheat bran medium with 75% moisture content. With lower (50%) and higher (85%) moisture levels, laccase activity was found to decrease significantly. If the amount of water is insufficient, diffusion of solvent and gas does not occur effectively and thus cell metabolism may slow down or stop. Low moisture content reduces nutrient diffusion, microbial coagulation, enzyme stability and substrate accessibility, whereas at high moisture level gas transfer is limited [14, 22]. Revankar et al. [20] tested the various moisture contents (40% -80%) and found that 70% was suitable for G. lucidum incubated on wheat bran medium.

3.4. Effect of inducers and additional substrates on laccase production

The effect of inducers and additional substrates such as soy flour, waste leaves, malt extract and copper on laccase production was tested. Soy flour is an important source of protein. In order to induce laccase activity, soy flour (SF) was added into the wheat bran (WB) medium at various ratios and the ratio of 1:1 induced the laccase production (Figure 4a). The production of laccase activity was only 2814 ± 105 U/L in the wheat bran medium but it increased to 4080 ± 826 U/L in the medium containing soy flour at a ratio of 1:1. Similarly, Aydinoglu and Sargin [23] reported the inducing effect of soy flour.

The effect of addition of various waste leaves into wheat bran media on laccase production of *G. lucidum* was also tested, but waste leaves did not show any positive effect on laccase production. Yeast extract is an organic nitrogen source and induces mycelial growth and also contains amino acids and vitamins which are effective in production and laccase synthesis [24, 25]. In our study, no inducing effect of yeast extract on laccase production was detected (Figure 4b). Negative effects of high yeast extract concentrations on enzyme production have also been reported by Mehta et al. [26].

Copper is an efficient inducer in laccase production. However, its inducing amount varies depending on the fungi or strain [5, 13, 27]. For this reason, the effect of various concentrations of copper on laccase production of this strain was also investigated. As shown in figure 4c, while the copper concentrations of 1 mM and 5 mM showed no inducing effect on laccase production, 10 mM copper concentration highly induced the laccase production and 1.8 times higher (5199 ± 133 U/L) laccase activity was determined in wheat bran medium with 10 mM copper than the activity determined in the medium without copper (2814 ± 105 U/L). Boran and Yesilada [5] reported that wheat bran media moistened with olive oil mill wastewater and vinasse containing copper have positive effect on laccase production of *Funalia trogii* and *Trametes versicolor*.

Zymogram study of the crude laccase source obtained from WB+SF medium supplemented with 10mM copper was also carried out by native polyacrylamide gel electrophoresis. The presence of laccase was investigated on gel by performing the activity staining with ABTS. Zymogram analysis showed only a single activity band for crude laccase (Figure 4d).

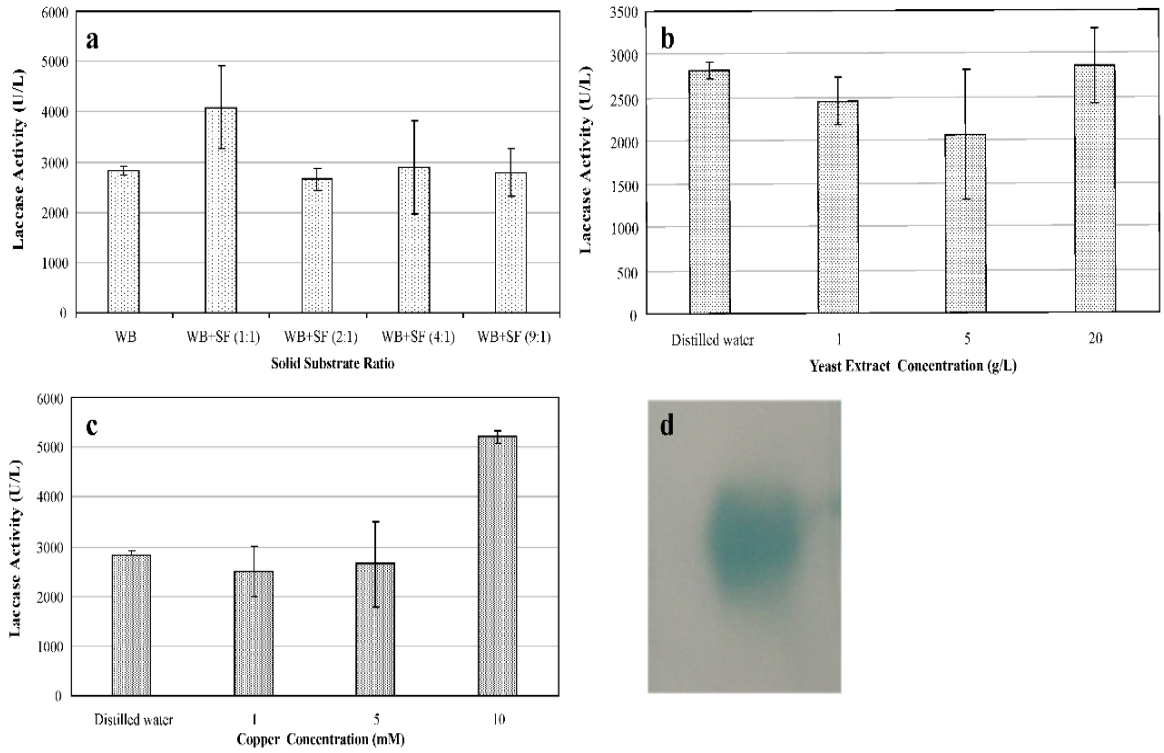

Figure 4. Effect of (a) solid substrate ratio (b) yeast extract concentration (c) copper concentration on laccase activity after 5 days of incubation and (d) zymogram of crude laccase. WB: Wheat bran, SF: Soy flour

3.5. SSF applications in tray type fermenter

Tray-type fermenter is commonly used in SSF processes [28]. This type of fermenter can be used for laccase production by filamentous fungi [29]. The laccase activity was 1582 ± 276 U/L in this medium moistened with distilled water without copper, but it was 2008 ± 826 U/L for cultures moistened with distilled water containing 10 mM copper.

3.6. Activity and stability studies by crude laccase of G. lucidum

Various factors such as pH and temperature effect the activity and stability of the enzymes. The efficiency of laccase enzyme in application depends on these factors. Therefore to detect the optimum temperature and pH values for enzyme activity is important. Firstly, the optimum temperature and pH values for crude laccase activity were determined. For this aim, the activities were determined at temperature ranges of 20-85 °C and pH 5.0. A continuous increase in enzyme activity was observed from 20 ºC to 70 ºC and the highest activity was detected at 70 ºC. While the laccase activity was 3546±173 U/L at 20 °C, it was increased remarkably to 12280±75 U/L at 70 ºC (Figure 5a). After 70 ºC, the activity of began to decrease. De Souza et al. (2002) reported that the crude laccase enzyme of *Pleurotus pulmonarius* is active at 50-55 °C. In another work, Stoilova et al. [30] obtained the highest activity for *Trametes versicolor* crude laccase at 45 °C. Yesilada et al. [31] reported the highest activity at 80-95 °C for crude laccase from *Funalia trogii* ATCC 200800. pH is an important factor for enzyme activity. Hence, the enzyme activity was also investigated at pH values between 2.0-7.0. As shown in figure 5b, the enzyme activity was high at pH values between 2.0-4.5 and then it decreased. Particularly, at values above pH 4.5, the activity was significantly reduced. Maximum pH activity was detected at pH 3 (Figure 5b).

Stability of the protein at high temperatures and different pH values is important for the use of enzymes in biotechnological and industrial processes [32]. Therefore, stability at high temperatures during industrial applications is the desired property of the enzymes [33]. Most of the white rot fungal laccases are active at 30-50 °C and rapidly lose their activity at temperatures over 60 °C [34]. Therefore, temperature and pH stabilities of this crude enzyme were also investigated (Figure 5c-d). The crude laccase enzyme retained about 100% of its activity for 30 min and 37 % for 120 min at 70 °C (Figure 5c). Ozsolen et al. [35] reported that the enzyme of *Trametes versicolor* ATCC200801 has lost its stability at temperatures above 50 °C. Yesilada et al. [31] found that crude laccase of *Funalia trogii* ATCC 200800 was stable about 2 h and 5 min at 60°C and 70°C, respectively. The crude enzyme obtained here, remained highly stable for 6 hours at all pH values (Figure 5d). Similarly, Yesilada et al. [31] reported that the enzyme activity of crude laccase from *Funalia trogii* ATCC 200800 was highly stable at all pH values (pH 3.0-9.0).

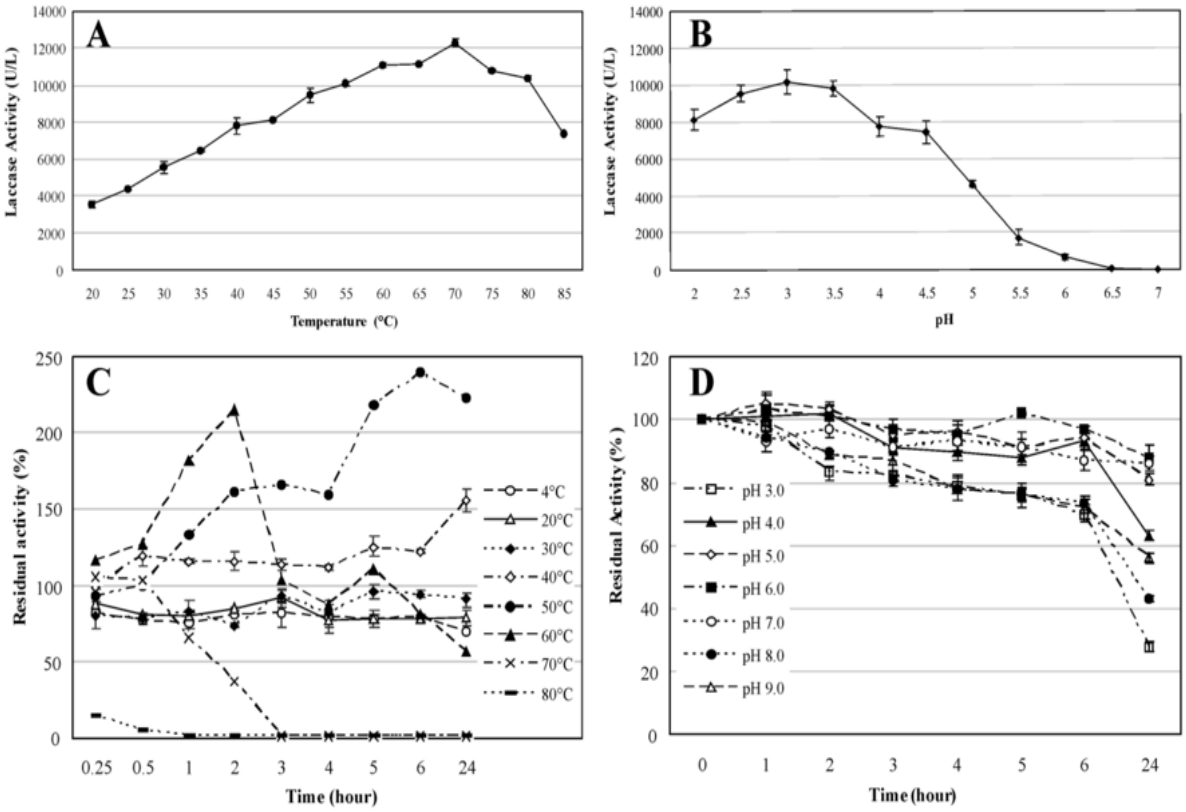

Figure 5. Effect of (a) temperature and (b) pH on activity and (c) temperature, (d) pH on stability of the crude laccase.

3.7. Dye decolorization activity by crude laccase of G. lucidum

The catalytic potential of enzyme source was very important for application. There are different studies on decolorization of various synthetic dyes with the enzymes of white rot fungi [36, 37]. Especially, laccase enzymes are used effectively in many color removal studies [10, 16]. For this reason, here, Remazol Brilliant Blue R (RBBR) (RB19), an anthraquinone dye, was chosen as the model dye to test catalytic property of this crude enzyme.

Firstly, the effect of the initial pH on decolorization of RB 19 was investigated. The best color removal as 22% was detected at pH 3.0 and 30 °C (Table 2). It has been reported that laccase of *G.*

lucidum is effective at acidic pH values [16]. Similarly, in our study, color removal was higher at low pH values (pH 2.5-4.0), and decolorization decreased at pH values above 4.0.

Table 2. Effect of pH and temperature on dye decolorization

		Dye decolorization (%) after:	
		60 sec	**300 sec**
pH values	**2.5**	9.10 ± 0.18	17.24 ± 0.61
	3.0	12.71 ± 0.43	22.38 ± 0.38
	3.5	11.13 ± 1.02	20.25 ± 0.77
	4.0	10.26 ± 0.19	17.02 ± 0.28
	4.5	9.64 ± 0.33	14.36 ± 0.47
	5.0	8.55 ± 1.04	11.06 ± 1.08
	5.5	8.59 ± 0.14	10.05 ± 0.51
	6.0	7.64 ± 0.33	8.01 ± 0.33
Temperature (°C)	**30**	12.71 ± 0.43	22.38 ± 0.38
	40	13.22 ± 0.35	28.98 ± 0.35
	50	14.91 ± 0.17	29.95 ± 0.21
	60	14.51 ± 0.20	18.82 ± 0.38

Initial dye concentration: 100 mg/L, Enzyme amount: 100 µL

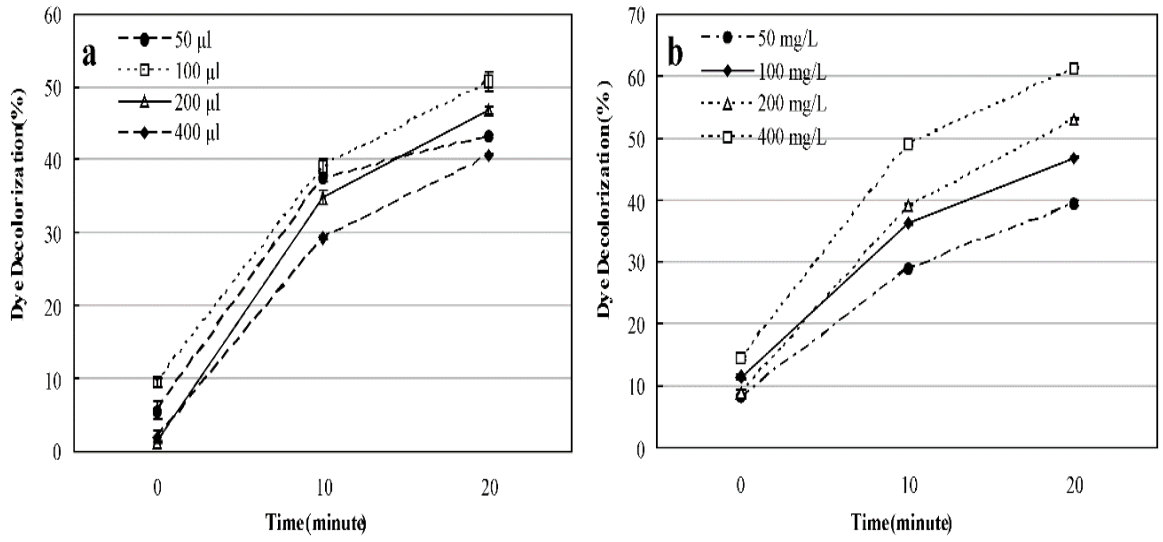

Figure 6. Effect of (a) enzyme amount and (b) dye concentration on decolorization.

The effect of temperature on decolorization was tested at pH 3.0 with 100 mg/L of dye and 100 µL of enzyme amount. As shown table 1, the decolorization activities obtained at 40 °C and 50 °C after 300 sec was 29% and 30%, respectively.

The effect of the crude enzyme amount and dye concentration on dye decolorization was also tested. The decolorization of the dye with the enzyme amounts of 50-400 µL was above 40%. The highest decolorization was obtained with 100 µL of crude enzyme (Figure 6a). Therefore, this enzyme amount was detected as the most effective enzyme amount for dye decolorization.

In order to test the effect of the dye concentration on decolorization efficiency, various dye amounts (50-400 mg/L) were incubated with 100 µL laccase enzyme amount at 40 °C and pH 3.0. It was observed that when the dye concentration increased, the efficiency of color removal increase. As shown figure 6b, the most effective color removal was found as 61% at dye concentration of 400 mg/L.

4. Conclusion

The newly isolated *G. lucidum* was detected as a good laccase producer under SSF condition. The produced laccase enzyme had high activity between pH 2.0 to 6.0 which the optimum was pH 3.0. It also showed high activity at 70 °C. This is important for applications where high temperature is needed. The crude laccase enzyme was stable at high temperature values such as 50 °C and this can make the process more advantageous in terms of biotechnological applications. It was possible to use it for decolorization of dyes such as RB 19 without any mediator. It is advantageous to use the crude enzyme in applications, without expensive purification processes.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Filiz Boran: Experimental stage, Conceptualization, Methodology, Writing - original draft, Investigation, Software, Data curation. Özfer Yesilada: Conceptualization, Visualization, Writing - original draft, Methodology.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

We thank to İnönü University Research Fund (Project no: 2010-118) for financial support of this study.

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